ISOLATION AND CHARACTERIZATION OF VESICLES INVOLVED IN HYPHAL TIP GROWTH OF *ACHLYA*

By

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ISOLATION AND CHARACTERIZATION OF VESICLES INVOLVED IN HYPHAL TIP GROWTH OF ACHLYA

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Achlya ambisexualis Raper is a heterothallic, oomycetous fungus. During sexual reproduction, hormone-induced male strains produce hyphal branches, which elongate by apical growth and differentiate into antheridia upon contact with the female. Antheridial hyphae presumably elongate by the same mechanism that permits vegetative growth. Theories have been proposed to account for the pattern of apical vegetative growth, and these involve the coordinated action of cell wall synthesizing enzymes (e.g., UDPG transferase) and cell wall hydrolyzing enzymes (e.g., cellulase) in the hyphal tip. The present study was conducted in order to gain information about the mechanism of vegetative hyphal tip growth in Achlya.

Some aspects of the growing and nongrowing states were examined. Mycelial growth is reduced by about 90% when mycelia are transferred from defined liquid medium (DLM) to 0.2% glucose medium (GM). Mycelia growing in DLM incorporate exogenous glucose into cell walls and secrete cellulase into the medium, but these processes are reduced by about 90-100% when mycelia are transferred to GM. Of the enzymes tested, only cellulase and alkaline phosphatase exhibit higher specific

activities in growing mycelia than in nongrowing mycelia; the specific activity of UDPG transferase does not change.

The enzyme cellulase exists both as a buffer-soluble and a buffer-insoluble form. The insoluble form is membrane-bound and can be solubilized with 1% triton X-100 or by incubation at room temperature in 0.5 mM DTT, with a subsequent 8- to 10-fold increase in activity. Freezing, sonication, and 1 M salts do not solubilize this cellulase. I conclude that this particulate cellulase is an integral membrane protein.

Mycelial homogenates were centrifuged isopycnically in a linear sucrose gradient, in which most of the cellulase activity equilibrates at a density of 1.19 g/cm^3. Most carbohydrate, UDPG transferase, IDPase, and ATPase also equilibrate here. Enrichment of these activities was achieved by recovering those particles that sediment from homogenates between 5,000 x g x 10 min and 25,000 x g x 10 min and recentrifuging them in a 15-35% sucrose velocity gradient, before a final isopycnic centrifugation in a linear 20-55% sucrose gradient. Particles equilibrating at 1.19 g/cm^3 consist of dictyosome cisternae and unidentified smooth membranes. The PTA-CrO_3 stain for plasma membranes fails to stain these particles; cisternae and some of the smooth membranes stain with the PASM stain for carbohydrate.

In order to identify cellulase-containing membranes, growing hyphae were examined electron-microscopically, and a number of cytochemical tests and ultrastructural enzyme localizations were performed. Hyphal tips contain cytoplasmic vesicles, which are apparently produced by dictyosomes. Vesicles are of at least two major classes, whose sizes are about 150 nm and about 80 nm in diameter, respectively. Some

vesicles in each class contain IDPase and the 150 nm vesicles stain with the PASM stain. Dictyosomes are IDPase-negative and PASM-positive. The plasma membrane and some of the 80 nm vesicles stain with the PTA-CrO_3 stain.

The identical distribution of cellulase, UDPG transferase, carbohydrate, and IDPase in isopycnic gradients indicates that transferase and cellulase are localized in the IDPase-positive, PASM-positive cytoplasmic vesicles. This supports the theory that postulates the coordinated involvement of cell wall synthesis and lysis in apical growth of fungi. The involvement of vesicles provides a mechanism for the simultaneous delivery of these materials to the apex. The fact that repression of growth is accompanied by a reduction of mycelial cellulase activity and abolition of cellulase secretion, while UDPG transferase activity is unchanged, supports the proposal that the rate of wall synthesis can be regulated by the availability of polysaccharide chain "primer" ends in the wall. Membrane-bound cellulase may be transferred to the cell surface by fusion of apical vesicles with the plasma membrane, at which time the enzyme is solubilized and produces cellulose "primer" ends in the wall by endohydrolysis. The subsequent release of cellulase into the medium may ensure that effective levels of activity occur only at the apex.

INTRODUCTION

Fungal hyphae are walled, filamentous cells, which extend by localized growth at their tips. This process includes continued biosynthesis of new cell walls. Cytological and biochemical studies of growing hyphae suggest that at least part of the synthetic process may involve apical vesicles derived from the Golgi apparatus, and attempts have been made to isolate these and other cellular structures in hopes of determining their contents. Among the proposed contents are wall synthesizing enzymes, wall hydrolyzing enzymes, and carbohydrate wall precursors; as yet, their simultaneous association with purified membranes of filamentous fungi has not been demonstrated.

The present study utilizes the fungus Achlya ambisexualis Raper in an examination of apical growth. The choice of the organism is appropriate because the involvement of the enzyme cellulase with a specific wall-related morphogenetic event in the life cycle is already well characterized, and a model employing cytoplasmic vesicles to explain this event has been proposed. Though the present study does not examine these events of hormone-induced branching, it is hoped that a better understanding of the mechanism of ordinary vegetative growth in Achlya will contribute to further studies both of hormone induction and of fungal growth in general.

REVIEW OF THE LITERATURE

Achlya ambisexualis Raper

The fungal species *Achlya ambisexualis* Raper is classified in the order Saprolegniales, class Oomycetes, division Mastigomycotina (Dick, 1973). Like other fungi, individuals exhibit an absorbtive, heterotrophic mode of nutrition plus a conspicuous cell wall and lack of motility in the vegetative state. Unlike most other fungi, however, asexual reproduction involves the production of heterokont zoospores, and the cell wall contains cellulose instead of chitin (Sparrow, 1960).

The growth form of *Achlya* is typical of most fungi. The body or thallus consists of branched cylindrical filaments called hyphae, and the assemblage of all the hyphae of a thallus is called a mycelium (Alexopoulos, 1962). Ordinarily, the mycelium is considered to consist of but one cell, which contains many nuclei and is not divided by cross walls. This is, perhaps, strictly true only of very young mycelia, because cross walls do form as the mycelia age. They function to wall off older, nonfunctional regions of cytoplasm and to delimit reproductive structures (Johnson, 1956). Thus, the vegetative portion of the mycelium, even in mature individuals, consists of from one to several extensively branched coenocytic cells, each capable of independent colonization, growth, and reproduction. In the case of water molds, there is evidence that, nutritionally at least, there is little

communication between widely separated parts of the same mycelium, reenforcing the impression of functional autonomy among growing regions (Jennings *et al.*, 1974).

Achlya is capable of both sexual and asexual reproduction. Asexually, individuals reproduce by fragmentation, by differentiation of hyphae into resistant gemmae, or by differentiation of vegetative apices into clavate zoosporangia (Johnson, 1956; Sparrow, 1960). Zoosporangial formation can be induced by depletion of nutrients (Klebs, 1899) and results in the formation of biflagellate spores, which settle and encyst after a period of swimming. Germination may then occur on a suitable substrate via a germ hypha (Coker, 1923).

Achlya ambisexualis is one of three *Achlya* species that are heterothallic; but, as the specific epithet implies, members of the species are ambivalent toward any rigid assignation of gender (Raper, 1951). Individuals exhibit varying degrees of sexual disposition, and many can be induced to act as either male or female, depending on the partners with which they are paired. In response to the proper hormonal cues, compatible vegetative thalli form appropriate sex organs *de novo*; their subsequent fusion is also under hormonal control (Raper, 1951; Barksdale, 1969). The best studied of the hormonal responses is that elicited by the steroid antheridiol, which is secreted constitutively by the vegetative female. Individuals capable of responding as a male exhibit a series of specific molecular responses to antheridiol induction (e.g., Kane *et al.*, 1973), culminating in the copious production of lateral antheridial branches (Barksdale, 1970). The branches grow toward the female thallus, where they make contact with oogonial initials.

Delimitation of sex organs, meiosis, gametogenesis, and fertilization follow (Raper, 1951).

It is the events between antheridiol uptake and lateral branch production that have attracted the most interest of Achlya researchers because the system provides a model system for investigating steroid hormone action. However, the steps of greatest significance to fungal biology are those of branch initiation itself, and the most relevant work here has dealt with the events of wall modification. At the time of branch initiation, the intracellular level of endocellulase (Cx) activity rises and then declines as the enzyme is secreted into the medium (Thomas and Mullins, 1967, 1969). Cellulase secretion and branch initiation are dependent on synthesis of RNA and protein and can be prevented by selective inhibitors (Kane et al., 1973; Horowitz and Russell, 1974; Timberlake, 1976). This response is seen only in those Achlya strains that produce antheridial branches and has led to the hypothesis that induction of antheridial branches requires the delivery of cellulase to specific points on the wall, where lytic wall thinning and softening permit a turgor-driven "blow out" of the wall (Thomas and Mullins, 1967, 1969).

Support for the above hypothesis derives from the observation that lateral walls are thinned at points of antheridiol-induced branch initiation, and these points are subtended by accumulations of cytoplasmic vesicles (Mullins and Ellis, 1974). In another investigation, hormone-induced hyphae were shown to contain vesicles beneath the lateral to subapical walls, which react positively to a cytochemical test for cellulase (Nolan and Bal, 1974). However, the vesicles in question were not associated with points of branch initiation, leaving open the

possibility that they were involved in some other cell process, such as ordinary vegetative growth.

It must, at this time, be emphasized that hormone-induced branching may not differ qualitatively from similar events during vegetative growth, inasmuch as branching is a regular part of fungal growth. In an induced thallus, branching increases, and growth occurs both in newly formed branches and in existing apices. Furthermore, after the initial modification of lateral walls for branch initiation, continued growth of antheridial hyphae presumably occurs by the same mechanism as that which permits vegetative growth. Knowledge of the method of vegetative fungal growth is, therefore, relevant to an understanding of reproductive growth.

Apical Growth and the Fungal Cell

Growth of filamentous fungal cells has been shown to occur by localized extension of the hyphal tips (Smith, 1923; Robertson, 1965). Regions more than about five micrometers from the tip do not elongate. New tips are, of course, initiated by branching in lateral regions, but subsequent growth is apical. Other forms of fungal growth are known, but they serve specialized functions, such as the elevation of terminal sporangia by intercalary growth of the sporangiophore (Castle, 1942). Apical growth is not unique to fungi but is a characteristic growth form of a variety of filamentous cells. Among these are algal filaments and rhizoids (Ott and Brown, 1974; Sievers, 1967) and pollen tubes and root hairs of higher plants (Rosen et al., 1964; Bonnett and Newcomb, 1966).

Because of its growth pattern, a fungal hypha consists of an apical region, which is constantly growing, and an older region, which was once the site of growth but is no longer capable of extension.

This dichotomy of growth potential is reflected in the cytoplasmic structure and chemistry of growing and nongrowing regions. Apical and mature regions have been shown to differ in their distributions of various macromolecules, enzyme activities, and reducing potential (Zalokar, 1965; Turian, 1976). Older regions of the hypha are typically highly vacuolate, and the cytoplasm is restricted to a thin, peripheral layer between the tonoplast and plasma membrane (Bracker, 1968). The cytoplasm contains a variety of eucaryotic organelles, which includes dictyosomes in the case of Oomycetes. Typically, the terminal 40-100 micrometers are nonvacuolate and are particularly rich in organelles. At the very apex, however, the cytoplasm is particularly devoid of most organelles and is occupied almost exclusively by a collection of apical vesicles (McClure, et al., 1968; Girbardt, 1969; Grove and Bracker, 1970; Grove et al., 1970). Based on this distribution, three distinct cytoplasmic zones are recognized, corresponding to the older vacuolate region, the subapical organelle-rich region, and the terminal vesiculate region (Grove et al., 1970).

The Fungal Cell Wall

In the mature cell walls of Oomycetes, typically from 80-90% of the dry weight is composed of carbohydrate, with protein and lipid constituting the rest; similar proportions are found in walls of other fungal groups (Bartnicki-Garcia, 1968). Analysis of Achlya cell walls reveals that the carbohydrate fraction contains from 10% to 15% cellulose in a weakly crystalline form (Parker et al., 1963; Aronson et al., 1967), while the remainder consists primarily of a highly branched glucan

containing β-1,3 and β-1,6 linkages (Aronson et al., 1967). Small amounts of nonglucose sugars are also present (Thomas, 1966; Dietrich, 1973).

In a manner resembling the walls of higher plants, these components are organized into what is essentially a biphasic system, which consists of a fibrillar phase enmeshed in an amorphous matrix phase (Preston, 1974). In fungi, however, the fibrillar elements are typically restricted to the inner part of the wall, so that the outer portion consists of amorphous materials only (Hunsley and Burnett, 1970). Recent work in which Oomycete cell walls were disassembled with specific enzymes has served to reveal the identity of some of the materials contributing to the various wall phases.

In Pythium acanthicum Drechsler, for instance, the outer layer was shown to be removable by laminarinase treatment, indicating a glucan with a high proportion of β-1,3- and β-1,6-linkages. Inner fibrils required treatment with both laminarinase and cellulase for complete dissolution, and the pattern of degradation indicated that these consisted of β-1,3- and β-1,6-glucan surrounding a weakly crystalline cellulose core (Sietsma et al., 1975). In Phytophthora parasitica Dastur, the outer matrix was also removable by laminarinase, though the matrix in the fibril layer required pronase treatment for removal. Microfibrils were completely removable with cellulase (Hunsley and Burnett, 1970; Hunsley, 1973). Thus, even within the Oomycetes, there seems to be room for considerable variation in cell wall architecture.

Cell Wall Modification During Growth

The importance of the cell wall in fungal morphogenesis is immense. The form of almost every fungal cell, and thus the function it performs, is determined by the structure of the cell wall. It is not surprising, therefore, that changes in fungal form (i.e., morphogenesis) usually involve cell wall modification in some way (Bartnicki-Garcia, 1968). Growth, the fundamental expression of morphogenesis, is no exception.

It is unarguable that a growing fungal cell must increase the area of its cell wall to accommodate the increase in volume of the cytoplasm; and this must be accompanied by wall synthesis, if growth is to continue indefinitely. Therefore, the prime concern of most models of fungal cell growth is the explanation of the development of the cell wall.

As was seen to be the case with the fungal protoplast, the fungal wall shows variations between growing and nongrowing regions of the same hypha. Lateral walls are typically thicker and possess more easily resolved layers than do apical walls; walls of P. parasitica vary in thickness from about 175 nm laterally to about 54 nm at the apex (Hunsley, 1973). Enzymatic disassembly reveals that there is only a small contribution of the outer amorphous glucan layer to the apical wall, and the apical fibrils are narrower and more loosely arranged than are lateral fibrils (Hunsley and Burnett, 1970; Hunsley, 1973). Further support for the gradation of materials is provided by reports of differential exposure of antigenic sites along a growing hypha (Fultz and Sussman, 1966; Hunsley and Kay, 1976) and differential staining with fluorescent brighteners (Gull and Trinci, 1974).

Because the apical wall is quite thin, one might expect this to be one of the weakest points of the wall, and this can be demonstrated by

immersing the hypha in dilute acidic solutions, which causes the cells to burst preferentially at the apex (Park and Robinson, 1966; Bartnicki-Garcia and Lipmann, 1972). Theories of wall development account for the differential osmotic stability by postulating that there exists in the apex a delicate balance between wall synthesis and wall lysis, which permits turgor-driven expansion of the plastic apical dome (Park and Robinson, 1966; Bartnicki-Garcia, 1973). Lateral rigidification could be accounted for by a predominance of synthesis in maturing regions (Bartnicki-Garcia, 1973) or by the activation of a secondary synthetic mechanism (Park and Robinson, 1966).

The requirement of turgor pressure for apical growth is demonstrated by cessation of hyphal elongation in hypertonic solutions (Robertson, 1965; Park and Robinson, 1966) and prevention of hormone-induced branching in Achlya by water stress (Thomas, 1970). Although turgor may provide no more than an expansive force for deformation of the plastic apex, contact between the plasma membrane and cell wall may also be required for activity of wall synthesizing enzymes (Shore and Maclachlan, 1975; Maclachlan, 1976) or for exocytosis (Robinson and Cummins, 1976). For example, hormone-treated Achlya hyphae under water stress fail to produce branches, and they accumulate intracellular cellulase, which is not secreted (J. T. Mullins, unpublished data).

Synthetic Processes

The need for wall synthesis in apical growth is, as stated, patent. The expectation of maximal activity in the apex stems both from poetic necessity and from autoradiography, which confirms that maximal deposition of wall precursors is in the hyphal tip (Gooday, 1971; McMurrough

et al., 1971). Enzymes capable of synthesizing products with the properties of wall polymers have been demonstrated both in fungi and higher plants.

For instance, a "soluble" cell fraction from Mucor rouxii (Calm.) Wehmer has been shown to contain the enzyme "chitin synthetase" in a zymogenic form that can be activated by a protease (Ruiz-Herrera and Bartnicki-Garcia, 1974; Ruiz-Herrera et al., 1975). Such synthetases have been found in several fungi of various groups (e.g., de Rousset-Hall and Gooday, 1975; Durán et al., 1975; Mills and Cantino, 1978), but that from M. rouxii is noteworthy because it is part of an enzyme complex borne by minute cytoplasmic particles termed "chitosomes" (Bracker et al., 1976). Apparently as a result of this association, not only is chitin synthesized de novo, but the resulting chains are also assembled into crystalline chitin microfibrils in vitro (Ruiz-Herrera and Bartnicki-Garcia, 1974; Ruiz-Herrera et al., 1975).

This is, unfortunately, not yet the case with the so-called "cellulose synthetases" and "glucan synthetases." These enzymes, though known in fungi (Wang and Bartnicki-Garcia, 1966; Meyer et al., 1976, Fèvre and Dumas, 1977), have been best characterized from higher plants, where they normally exhibit the ability to transfer a limited number of radioglucose molecules from nucleoside-diphosphoglucose to an endogenous acceptor (for references, see Preston, 1974). Furthermore, enzyme preparations are usually crude, and the products in vitro are often heterogeneous and very much a function of assay conditions (Ordin and Hall, 1968). For these reasons, it may be better to refer to these enzymes as "transferases," reserving the term "synthetases" until their roles in vivo are more certainly known.

Lytic Processes

Evidence for the involvement of lytic forces in apical growth is based in part on the already mentioned plasticity of the apical wall, as demonstrated by osmotic rupture. The involvement of specific wall-hydrolytic enzymes in distinct morphogenetic events of fungi and higher plants is well documented and includes such phenomena as leaf abscission (Lewis and Varner, 1970), ripening of fruits (Hall, 1964), autolysis of Coprinus fruiting bodies (Iten and Matile, 1970), colonial growth morphology of Neurospora (Mahadevan and Mahadkar, 1970), antheridiol-induced branching in Achlya (Thomas and Mullins, 1967, 1969), and fruiting in Schizophyllum (Wessels, 1966).

The involvement of specific hydrolases with ordinary vegetative growth has been indicated in studies of both fungi and higher plants. The enzyme cellulase is secreted by growing hyphae of A. ambisexualis and accumulates in the medium (Thomas and Mullins, 1969). Its function is apparently not nutritional because mycelia are incapable of using cellulosic substrates as carbon sources (Thomas, 1966). And, in a related fungus, Saprolegnia monoica Pringsheim, the activity of intracellular cellulase was found to be highest in regions of the colony nearest the growing edge (Fèvre, 1977).

A number of manipulations of growing cells elicit responses that can be interpreted as the result of disturbances in the presumed balance between synthesis and lysis. Exposure of pollen tubes to exogenous hydrolases can enhance the rate of growth (Roggen and Stanley, 1969), and Neurospora hyphae can be induced to branch by similar treatments (de Terra and Tatum, 1961). Uptake of cholesterol by Pythium hyphae results in decreased levels of wall hydrolases and corresponding morphological

aberrations (Sietsma and Haskins, 1968), and yeast cell walls lyse upon exposure to 2-deoxyglucose, presumably because wall synthetases are inhibited (Johnson, 1968).

The distribution of cellulase in the pea epicotyl also suggests a relationship between vegetative growth and "the delicate balance." Although synthetase activity is about equal in both growing and nongrowing regions of the stem, the cellulase activity is high in actively growing tissues and absent elsewhere (Maclachlan, 1976). Furthermore, auxin treatment increases cellulase activity of decapitated epicotyls beyond the pretreatment level (Fan and Machlachlan, 1966), whereas net synthetase activity is merely maintained (Ray, 1973; Spencer et al., 1971).

Other cases in which high levels of wall hydrolases are associated with growth include the budding stages of yeast, which exhibit high activities of protein disulfide reductase (Nickerson and Falcone, 1959) and β-glucanase (Cortat et al., 1972).

An exact role for degradative enzymes in wall biosynthesis is not readily apparent. In their simplest role, hydrolases might be involved in mere wall loosening, which could permit the passive extension of the wall by turgor. Loosening can be demonstrated by treating isolated cell walls with exogenous polysaccharidases to increase their extensibility (Olson et al., 1965). In higher plants, however, evidence seems to argue against a role of polysaccharidases as regulators of extension (Cleland, 1968; Ruesink, 1969). Instead, current interest is on the potential for hydrogen ions to disrupt hydrogen bonds between cellulose elementary fibrils and attached matrix polysaccharides (Keegstra et al., 1973; Davies, 1973) or the polypeptide "extensin," which may represent

a covalently bonded, selectively cleavable linker between polysaccharide chains (Lamport, 1974).

In the case of the enzyme cellulase, an indirect, though critical, role in wall development may lie in its ability to generate free cellulose chain ends by endohydrolysis. According to Maclachlan (1976), the availability of cellulose "primer" ends is a rate limiting step in cellulose biosynthesis, and the rate of synthesis should be increased by pretreatment or cotreatment of walls with cellulase. The expected enhancement has been demonstrated using pea epicotyl segments preincubated with either fungal cellulases (Maclachlan, 1976) or native pea cellulases (Wong et al., 1977a). Proof that endogenous cellulases actually function this way in vivo is lacking, though the relative distributions of cellulase and synthetase activities in pea epicotyls is consistent with this explanation (Maclachlan, 1976).

Involvement of Cytoplasmic Structures in Wall Formation

Morphological Evidence

Models assigning roles to cellular components in hyphal growth have relied heavily on the evidence provided by electron microscopy of hyphal tips. Although there are taxonomically related variations in the distribution of organelles in fungal tips, vesicles are present in hyphal apices of all growing fungi (Grove and Bracker, 1970; Bartnicki-Garcia, 1973). Vesicles are also associated with the tips of basidial sterigmata (McLaughlin, 1973), buds of yeast (Moor, 1967; Sentandreu and Northcote, 1969), germinating spores (Bracker, 1971), fungal rhizoids (Barstow and Lovett, 1974), and apically growing structures of algae and

higher plants (Rosen et al., 1964; Bonnett and Newcomb, 1966; Ott and Brown, 1974).

Vesicles often contain fibrous materials, which react with cytochemical stains for carbohydrates (Heath et al., 1971; Dargent, 1975; Meyer et al., 1976). Their apparent carbohydrate content and apical location suggest that they may contribute their contents to the growing cell wall (Grove et al., 1970). The origin and fate of wall vesicles cannot be proven from fixed material, but there is sufficient morphological evidence to suggest that apical vesicles arise from dictyosomes or their equivalents and secrete their contents into the cell wall (Grove et al., 1970).

The involvement of dictyosomes in secretion is exhaustively documented in a number of plant and animal systems (e.g., Mollenhauer and Morré, 1976; Palade, 1975). According to the theory of Palade (1975), secretory products are sequestered within membrane-delimited spaces and transferred through the endomembrane system (including the Golgi apparatus) to the extracellular mileau by exocytosis. The current model of apical growth as a secretory event involving exocytosis of Golgi-derived apical vesicles is consistent with that theory. Evidence for exocytosis is tenuous, of course, but support derives from electron microscopical images that seem to show apical vesicles in a state of fusion with the plasma membrane (Grove et al., 1970; Bracker, 1971); these images resemble those seen in more rigorously documented examples of exocytosis, such as mucocysts of Tetrahymena (Satir et al., 1973). This would result in a release of vesicle contents into the wall and would have the added virtue of contributing vesicle membrane to the plasma membrane.

Theories proposed to explain the direction and motive force for vesicle migration toward the apex have taken into account the already mentioned gradients of enzyme activity and reductive potential observed in hyphal tips; a result might be an electrochemical gradient sufficient to account for vesicle migration by "electrophoresis" (Bartnicki-Garcia, 1973). An explanation with more experimental support requires the action of contractile cytoplasmic microfilaments; cytochalasin B, which disrupts microfilaments, prevents apical growth in root hairs (Franke et al., 1972) and pollen tubes (Mascarenhas and Lafountain, 1972).

Biochemical Evidence

On morphological and theoretical grounds, vesicles associated with a number of apically growing systems have been suggested to contain a variety of materials that are consistent with a role in wall synthesis. Among these are carbohydrate wall precursors and presynthesized wall components (Larson, 1965; Seivers, 1967; McClure et al., 1968; Grove et al., 1970; Bartnicki-Garcia, 1973), wall synthesizing enzymes (Grove et al., 1970; Bartnicki-Garcia, 1973), and wall softening enzymes (Moor, 1967; Girbardt, 1969; Grove et al., 1970; Bartnicki-Garcia, 1973). In addition, other enzyme activities have been indicated by in situ cytochemical tests (Dargent, 1975; Meyer et al., 1976). However, electron microscopical evidence can only be confirmed by actual isolation and biochemical analysis of apical vesicles.

Filamentous fungi

There are reports of attempts to isolate apical vesicles from three fungi. The first, in Gilbertella persicaria (Eddy) Hesseltine, is unsubstantiated by published data and reports the recovery of subcellular

particles that contain polysaccharides composed of sugars characteristically found in the cell wall (Grove et al., 1972). In Phytophthora palmivora Butler (Meyer et al., 1976), it was shown that "UDPG transferase" activity is associated with cell walls and with a membrane fraction that may contain vesicles derived from the endoplasmic reticulum (ER). Exo-β-1,3-glucanase is generally associated with a second membrane fraction which may contain Golgi vesicles. In Saprolegnia monoica (Fèvre and Dumas, 1977), "glucan synthetase" activity is associated with a crude "wall" fraction and with membrane fraction that apparently contain both dictyosome cisternae and unidentified membranes. These fractions also contained β-1,3-glucanase and cellulase activities (Fèvre, 1977). Convincing correlations between these materials and distinct classes of subcellular particles is lacking in reports on all three fungi, and biochemical support for the involvement of specific organelles in tip growth is, at this time, largely by analogy to other, better characterized systems in yeast and higher plants. These will now be discussed.

Pollen tubes

A higher plant cell with apical growth, which has been biochemically investigated, is the pollen tube. Here, work has largely been restricted to identification and labeling of carbohydrates in walls and membrane fractions. Membranes obtained from Lilium pollen contain carbohydrates characteristic of the wall matrix (Van Der Woude et al., 1971), and the kinetics of labeling with radioactive precursors indicates that this material eventually contributes to the tube wall (Morré and Van Der Woude, 1974). Morphological evidence indicates that these

membranes are derived from dictyosomes and/or endoplasmic reticulum (Van Der Woude and Morré, 1968; Van Der Woude et al., 1971).

A noteworthy report is that of Engels (1973, 1974) who isolated from petunia pollen a fraction of membranes that he identified as Golgi vesicles using morphological criteria. On the basis of X-ray diffraction spectra, one component of the carbohydrate in these vesicles was identified as a mixture of cellulose I and cellulose II (Engels, 1974). This is the only report of true cellulose in cytoplasmic particles of a higher plant. Despite numerous investigations in other organisms, the only other report of *in vivo* cellulose synthesis in an intracellular compartment is a report of the unusual wall of the alga *Pleurochrysis* (Brown et al., 1970). Here the wall is composed of overlapping cellulosic scales, which are apparently preassembled entirely within dictyosomes and exported in vesicles to the cell surface.

Yeast cells

In yeast, investigations have focused primarily on the assembly and modification of the mannoprotein component of the wall matrix. Evidence from the dimorphic fungus *M. rouxii* has implicated this glycoprotein in the control of yeast morphogenesis, and the enzyme disulfide reductase is postulated to soften the wall by cleaving disulfide bridges between mannoprotein molecules (Bartnicki-Garcia and Nickerson, 1962).

The critical components of the yeast endomembrane system are the ER, cytoplasmic vesicles, and the plasma membrane; yeasts lack dictyosomes, and the vesicles are believed to be produced directly by the ER (Moor, 1967). Evidence indicates that all these compartments are involved in the assembly of mannoprotein and in subsequent modification of

these and other wall components (Matile et al., 1971; Cortat et al., 1972, 1973). Autoradiography shows initial mannose incorporation to be intracellular (Kŏsinová et al., 1974), and the results of cell fractionations indicate that the mannan chain is assembled sequentially, with the first sugar incorporated in the ER and subsequent sugars incorporated as the complex moves through the vesicles to the plasma membrane (Lehle et al., 1977). Other materials which may be involved in wall metabolism have also been detected in yeast cell particles, including β-1,3-glucanase, which is present in ER, vesicle, and plasma membrane fractions (Matile et al., 1971; Cortat et al., 1972). Wall fibril synthesis appears to be a function of the cell surface and is regulated independently of matrix synthesis, which can be preferentially uncoupled by cycloheximide (Nečas, 1971).

Formation of the chitinous yeast septum appears to involve at least two cellular compartments. Chitin synthetase is attached to the inner side of the plasma membrane as an inactive zymogen (Durán et al., 1975). The septum begins to form after a proteolytic activator is delivered in vesicles to the appropriate sites on the plasma membrane (Cabib and Farkaš, 1971).

Higher plants

The contribution of internal membranes to cell wall synthesis is perhaps best demonstrated in the auxin-stimulated pea epicotyl. The work of Peter Ray and collaborators has demonstrated the role of the Golgi apparatus in the synthesis of matrix materials and in their subsequent transfer to the wall (Ray et al., 1969, 1976; Robinson and Cummins, 1976; Robinson et al., 1976). Polysaccharides labeled in vivo with radioglucose are associated both with dictyosome cisternae and dictyosome

vesicles. Intracellular synthetase activity is associated with the cisternae only (Ray et al., 1976). The in vivo labeled products contain polysaccharides with linkages characteristic of the wall matrix, and only 3% to 8% of the linkages are β-1,4-glucan. Pulse-chase experiments demonstrate that labeled materials are transferred from dictyosome cisternae to vesicles and ultimately to the wall (Robinson et al., 1976). Further work by Maclachlan and coworkers (Shore and Maclachlan, 1975; Shore et al., 1975) supports the concept of matrix formation by dictyosomes, as does that by Harris and Northcote (1971), who worked with pea roots.

The site of microfibril (cellulose) synthesis in higher plants has not been biochemically defined, but evidence continues to indicate the plasma membrane-cell wall interface (Preston, 1974). Synthetase activity capable of generating β-1,4-glucan linkages can be detected in isolated dictyosomes (Ray et al., 1969), smooth ER (Shore and Maclachlan, 1975), and plasma membrane fractions (Van Der Woude et al., 1974), but this can account for no more than about 5% of the in vivo rate of cellulose synthesis (Maclachlan, 1976). Furthermore, since most reports indicate that, in vivo, only matrix polysaccharides are synthesized intracellularly, cellulose synthetase activity in these membranes is interpreted either as activity of enzymes in transit to the wall (Shore and Maclachlan, 1975) or as activity required to produce the β-1,4 linkages found in certain matrix components (Ray et al., 1976).

In higher plants, cellulose has not been reported within vegetative cells using biochemical criteria, which suggests its assembly at the cell surface (but cf. Brown et al., 1970; Engels, 1974). In addition, autoradiographic data from sycamore seedlings indicate that, under

conditions of maximal cellulose synthesis and minimal matrix synthesis, most activity is associated with the plasma membrane-cell wall interface (Wooding, 1968). That the interface itself is critical to synthesis is demonstrated by the reduction of incorporation upon plasmolysis (Maclachlan, 1976) or physical disruption (Shore and Maclachlan, 1975). Current attention has been directed toward plasma membrane particle complexes postulated by Preston (1974) and which may have been demonstrated recently in corn (Mueller et al., 1976).

Cellulases, which have been implicated in wall synthesis (Wong et al., 1977a), are also associated with subcellular compartments, including the cell wall and endoplasmic reticulum in peas (Bal et al., 1976) and plasma membrane in kidney beans (Koehler et al., 1976). Associations with dictyosomes have not been reported.

In summary, models of hyphal tip growth employ the coordinated activities of wall degradation and wall synthesis. These processes would require specific enzymes, wall precursors, and a mechanism for their simultaneous delivery to the apex. Morphological studies indicate that Golgi-derived apical vesicles are involved. Furthermore, evidence from other systems in which cytoplasmic vesicles play a role in wall metabolism lends support to models assigning a similar role to vesicles in hyphal growth.

MATERIALS AND METHODS

General Culture Methods

An isolate of Achlya ambisexualis Raper, strain E87, was provided by Dr. J. T. Mullins; derivation of the strain is described by Barksdale (1960). Stock cultures were maintained on YPSS agar slants (Emerson, 1941) at 5 C.

Mycelia were grown in a defined liquid medium (DLM) (Mullins and Barksdale, 1965) of the composition given in Table 1. The inoculum was obtained by a modification of the method of Griffin and Breuker (1969). A small amount of mycelium was transferred from the YPSS slant to a petri plate containing "enriched medium" (Kane, 1971), which contains the same ingredients as DLM, except monosodium-ℓ-glutamate, 3.0 mM; D-glucose, 77.7 mM; casein hydrolysate, 0.15% w/v; agar, 2.5% w/v. After two days' growth at 24 C, a plug was removed from the center of the colony with a #10 cork borer and cut into about 9 pieces. These were washed in 50 ml of sterile 0.5 mM $CaCl_2$ in a 250 ml flask for 2 hr at 24 C on a shaker set at 100 rpm. The liquid was decanted and replaced with another 50 ml volume of sterile 0.5 mM $CaCl_2$, and the flask was returned to the shaker. After 15 to 20 hours, the sample was removed aseptically, and the zoospores and spore cysts were counted on a hemacytometer. A volume of liquid containing 200,000 spores was transferred to 200 ml of DLM in a 500 ml Erlenmeyer flask. This was incubated under the same conditions as those used to obtain the spore

TABLE 1. The composition of Defined Liquid Medium (DLM) (modified from Mullins and Barksdale, 1965)

Monosodium-ℓ-glutamate	2.4 mM
D-glucose	11.1 mM
Tris·SO_4 buffer, pH 6.9	10.0 mM
ℓ-methionine	0.1 mM
KCl	2.0 mM
$MgSO_4$	0.5 mM
$CaCl_2$	0.5 mM
HEDTA	72.0 µM
KH_2PO_4	1.5 µM
$Fe(NH_4)_2(SO_4)_2$	36.0 µM
$ZnSO_4$	15.0 µM
$MnSO_4$	9.0 µM
Sulfosalicylic acid	46.0 µM

inoculum. After a 24 hr lag, mycelial fresh weight (FW) increased to about 8 g per flask at about 72 hr. Accordingly, cultures were harvested at about 48 hr, which represented the midpoint in the growth curve and yielded about 5 g FW per flask. Harvest was accomplished by pouring the contents of each flask into a funnel lined with miracloth (Chicopee Mills, Inc.).

Cell Homogenization

Disruption was accomplished by grinding mycelia in a mortar with 5 g of acid-washed sea sand. The amount of mycelium and the composition of the homogenizing solution varied with certain experiments, and these details will be specified with the descriptions of the individual experiments. Generally, the homogenizing solution contained 0.03 M tris·HCl buffer (pH 7.6 measured at RT) and 30% w/w sucrose. In certain experiments, 15 mM dithiothreitol (DTT) and/or 0.3% bovine serum albumin (BSA) were included. Mycelia were ground for 30 sec at 5 C, and the homogenate was filtered through miracloth. The retained material was rehomogenized for 15 sec in a small volume of homogenizing solution which had been diluted until the sucrose concentration was 10% w/w. The second homogenate was refiltered on the same piece of miracloth, and the retained material was discarded.

Centrifugations

Isolation of cell particles was affected using either of two centrifuges. The Sorvall RC-2B high speed centrifuge with an SS-34 rotor was used for all differential centrifugations of homogenates. The Beckman L2-65B preparative ultracentrifuge with an SW 27 or SW 27.1

horizontal rotor was used for all gradient centrifugations, and sedimentation of gradient fractions was achieved with the 65 rotor.

In addition, the Sorvall GSA rotor was used in the recovery of ethanol-insoluble cellulase from culture filtrates.

Assays

Unless otherwise stated, all substrate biochemicals were obtained from Sigma Chemical Co., St. Louis, Mo. All pH's were measured at room temperature with a "tris" electrode (Sigma Chemical Co.). All spectrophotometry was performed with a Gilford model 240 spectrophotometer.

Bio-Rad Assay for Protein (Bio-Rad Technical Bulletin, 1977)

Five milliliters of Bio-Rad dye reagent were added to 50-200 µl of protein sample and mixed. After 10 min, absorbance at 595 nm was read. Standards were made with Bovine Serum Albumin (BSA).

Anthrone Test for Carbohydrate (Herbert et al., 1971)

Anthrone reagent:

anthrone	200.0 mg
absolute ethanol	5.0 ml
75% v/v H_2SO_4	95.0 ml

Five milliliters of cold (4 C) anthrone reagent were added to 1.0 ml of cold sample. The mixture was heated at 100 C for 10 min, and absorbance was read at 625 nm. Standards were made with glucose.

Phenol Test for Carbohydrate (Herbert et al., 1971)

Reagents:

5% w/v phenol

concentrated H_2SO_4

One milliliter of phenol and 5.0 ml of H_2SO_4 were added to 1.0 ml of aqueous sample. After cooling, absorbance was read at 488 nm. Standards were made with glucose.

Ferrous Sulfate-Ammonium Molybdate Assay for Inorganic Phosphorus (modified from Taussky and Shorr, 1953)

Reagent:

$FeSO_4 \cdot 7H_2O$	5.0 g
$(NH_4)_6MoO_{24} \cdot 4H_2O$ (10 mg/ml in 10 N H_2SO_4)	10.0 ml
distilled water	85.0 ml

Two milliliters of reagent were added to 250 µl of sample. Absorbance at 710 nm was read after 10 min. Standards were made with KH_2PO_4.

Acid Phosphatase (EC 3.2.3.2) (modified from Ray et al., 1969)

Substrate:

$MgCl_2$	2.2 µmol
p-nitrophenyl phosphate	0.8 mg
80 mM citrate buffer, pH 5.0	1.0 ml

Fifty to one hundred microliters of sample were added to 50 µl of substrate and incubated for 30 min at 37 C. The reaction was terminated by adding 2.0 ml of 0.2 M Na_2CO_3, and absorbance was read at 410 nm. A molar extinction coefficient for p-nitrophenol of 18,000 was assumed (Meyer, 1976).

Adenosine Triphosphatase (ATPase) (EC 3.6.1.3) (modified from Marriott, 1975)

Substrate:

tris·ATP	1.5 µmol
$MgCl_2$	5.0 µmol
16.7 mM tris·HCl buffer, pH 7.2	1.0 ml

Fifty to one hundred microliters of sample were added to 500 µl of substrate. Two hundred fifty microliters were removed and assayed for P_i. The remainder was incubated at 37 C for 4 hr, then 250 µl were removed and assayed for P_i released.

Alkaline Phosphatase (EC 3.1.3.1)

Substrate:

$MgCl_2$	2.0 µmol
p-nitrophenyl phosphate	0.8 mg
50 mM tris·HCl buffer, pH 9.0	1.0 ml

One hundred microliters of sample were added to 500 µl of substrate. The mixture was incubated 4 hr at 37 C, and 2.0 ml of 0.2 M Na_2CO_3 were added to dilute the mixture to a volume readable in the spectrophotometer. Absorbance was read at 410 nm.

Cellulase (EC 3.2.1.4) (modified from Bell et al., 1955)

Substrate:

carboxymethyl cellulose (type 7Mf, Hercules Powder Co.)	12.0 g
merthiolate	0.5 g
0.018 M sodium citrate buffer, pH 5.0	1.0 l

One milliliter of sample was added to a size "300" Ostwald-Fenske viscometer tube containing 5.0 ml of substrate, which had equilibrated at 30 C. After mixing, the flow time was measured at T_0 and after various intervals, and the difference in flow time was determined.

Cytochrome Oxidase (EC 1.9.3.1) (modified from Hodges and Leonard, 1974)

Substrate:

cytochrome c (Sigma type III, from horse heart, oxidized form)	11.0 mg
50 mM potassium phosphate buffer, pH 7.5	60.0 ml

Cytochrome c solution is reduced chemically with sodium dithionite.

Twenty to one hundred microliters of sample were added to 2.0 ml of substrate in a spectrophotometer cuvette. The change in absorbance at 550 nm was monitored during the phase of linear change. A molar extinction coefficient of 18,500 was assumed for cytochrome c.

Glucose-6-phosphatase (EC 3.1.3.9) (modified from Hübscher and West, 1965)

Substrate:

EDTA	4.0 µmol
KF	2.0 µmol
glucose-6-phosphate	6.8 mg
0.4 M sodium "PIPES" buffer, pH 6.5	1.0 ml

Fifty to one hundred microliters of sample were added to 500 µl of substrate, and 250 µl were removed for assay of P_i. The remainder was incubated for 4 hr at 37 C, and 250 µl were removed for assay of P_i released.

β-glucosidase (EC 3.2.1.21) (modified from Parish, 1975)

Substrate:

p-nitrophenyl-β-D-glucoside	0.5 mg
0.1 M sodium citrate buffer, pH 5.0	1.0 ml

Fifty to one hundred microliters of sample were added to 500 µl of substrate. The mixture was incubated for 30 min at 37 C, and the reaction was terminated with 2.0 ml of 0.2 M Na_2CO_3. Absorbance was read at 410 nm, and a molar extinction coefficient for p-nitrophenol of 18,000 was assumed.

Inosine Diphosphatase (IDPase) (modified from Ray et al., 1969; Shore and Maclachlan, 1975)

Substrate:

$MgCl_2$	1.0 µmol
inosine diphosphate	1.4 mg
0.1 M tris·HCl buffer, pH 7.5	1.0 ml

One hundred microliters of sample were added to 500 µl of substrate, and 250 µl were removed for assay of P_i. After incubation of the remainder for 4 hr at 37 C, 250 µl were removed for assay of P_i released.

UDPG transferase (EC 2.4.1.12) (modified from Ray et al., 1969; Shore and Maclachlan, 1975)

Substrate:

Solution I:	UDPG	925 µg
	^{14}C-UDPG (250 µCi/µmol in 1.0 ml, New England Nuclear)	100 µl
	distilled water	2.0 ml

Solution II:	$MgCl_2 \cdot 6H_2O$	68 mg
	cellobiose	51 mg
	dithiothreitol (DTT)	8.0 mg
	0.2 M sodium phosphate buffer, pH 5.8	10.0 ml

One hundred microliters of solution I, 100 µl of solution II, and 100 µl of sample were added to a 15 ml conical glass centrifuge tube. (The final concentration of reagents was UDPG, 0.242 mM; cellobiose, 5 mM; $MgCl_2$, 11 mM, DTT, 1.7 mM; buffer, 67 mM. The reaction vessel contained 7.27×10^{-8} moles of UDPG with 119 nCi of radioactivity.) After incubation at room temperature for 20 min, the reaction was terminated by adding 5.0 ml of 70% v/v ethanol. About 30 mg of powdered Whatman cellulose were added, and the tube was centrifuged in an IEC clinical centrifuge at about 1,000 x g for 5 min. The supernatant was discarded, and the sediment was washed three more times in 70% ethanol (15.0 ml total volume). Excess ethanol was permitted to evaporate from the final pellet, which was then resuspended in 3.0 ml of distilled water for liquid scintillation counting.

Liquid Scintillation Counting

Radioactive materials in 3.0 ml of water were transferred to a glass scintillation vial, and 5.0 ml of PCS scintillation fluid (Amersham Searle Co.) were added. The sample was shaken to form a gel and to disperse any solid materials, and radioactivity in the vials was measured with a Packard Tri-Carb Liquid Scintillation Spectrometer, model 3385. Results in counts-per-minute were converted to decays-per-minute

(dpm) using the channels ratio method, and μCi of radioactivity present was calculated, assuming 2.22×10^6 dpm/μCi. For some experiments, substrate incorporation (in moles) was calculated by isotope dilution.

Statistical Methods

When sufficient data permitted, values were reported with the associated standard deviation, which was calculated as the square root of the sample variance. When possible, comparisons between two samples are accompanied by a figure representing the degree of confidence in their statistical difference. This was calculated by a "two-tail" Student's "t" test. (All methods are from Runyon and Haber, 1971).

Electron Microscopy

Hyphae or subcellular fractions (obtained by methods to be described later) were fixed for 30 min at room temperature with 4% v/v glutaraldehyde in 0.05 M sodium cacodylate buffer, pH 7.2. After rinsing in several changes of buffer, the material was postfixed in 1% w/v OsO_4 in 0.05 M cacodylate buffer, pH 7.2, for 30 min at room temperature. Samples were again washed several times in buffer and dehydrated in a graded series of ethanol washes, terminating in absolute acetone. Material was infiltrated with an epoxy embedding medium (Table 2) and polymerized at 60 C for 24 to 48 hr in a flat embedding mold. Embedded samples were mounted on metal microtome stubs and sectioned on a Sorvall Porter-Blum MT-2 ultramicrotome. Thin sections were poststained for 5 min with lead citrate (Reynolds, 1963) or with 1% $BaMnO_3$ before examination with a Hitachi HU-11C or HU-11E electron microscope.

On some samples, cytochemical tests were performed, and this necessitated various modifications of the above scheme.

TABLE 2. Embedding medium for electron microscopy

Reagent	Manufacturer	Amount
Epon 812	Polyscience, Inc.	12.5 g
Araldite 6005	R. P. Cargille Lab., Inc.	11.5 g
DDSA	Tousimis Research Corp.	25.5 g
BDMA	Ladd Research Ind., Inc.	0.14 ml/10 g*

* BDMA is added just before use.

Cytochemical Tests

Phosphotungstic Acid-Chromic Acid ($PTA\text{-}CrO_3$) Stain (Roland et al., 1972)

Staining reagent:

phosphotungstic acid	0.1 g
CrO_3	1.0 g
distilled water	10.0 ml

Thin sections carried in polyethylene rings were oxidized by flotation on 1% w/w periodic acid for 30 min at room temperature. After washing by flotation on distilled water, sections were floated on $PTA\text{-}CrO_3$ staining reagent for 5 min at RT and rinsed on distilled water. Sections were mounted on grids for viewing without poststain.

Periodic Acid-Silver Methenamine (PASM) Stain (Martino and Zamboni, 1967)

Staining reagent:

hexamethylene tetramine	90 mg
$AgNO_3$	10 mg
4 mM sodium borate buffer, pH 9.0	10 mg

Thin sections in polyethylene rings were preoxidized in acidic H_2O_2 (15% H_2O_2 in 2% HCl) for 30-60 min at RT to remove osmium stain. After rinsing, sections were further oxidized in 1% w/w periodic acid for 15-30 min at RT and rinsed. Staining was performed by incubating oxidized sections for up to 2 hr in staining reagent at 60 C, followed by a distilled water rinse. After incubating in 1% v/v Kodak photographic fixer for 5 min at RT, sections were rinsed and mounted on grids for viewing.

Cytochemical Localization of IDPase (modified from Novikoff and Goldfischer, 1961)

Substrate:

IDP	1.0 mg
$MnCl_2$	1.0 mg
$PbNO_3$	12.0 mg
0.4 M tris·HCl buffer, pH 7.2	10.0 ml

Hyphae were fixed by the standard method and washed in cacodylate buffer, followed by 0.5 M tris·HCl buffer, pH 7.2. The material was then incubated in substrate solution for 60 min at 37 C and rinsed in tris buffer. Samples were postfixed and embedded as before, and thin sections were stained with lead citrate before viewing.

Cytochemical Localization of Alkaline Phosphatase (Hugon and Borgers, 1968)

Substrate:

Na-β-glycerophosphate	25.0 mg
$PbNO_3$	13.0 mg
0.04 M tris·maleate buffer, pH 9.0	10.0 ml

Hyphae were fixed and rinsed in cacodylate buffer, followed by 0.05 M tris·maleate buffer, pH 9.0. Incubation in substrate solution followed for 30-60 min at 37 C; and hyphae were washed, postfixed, and dehydrated in the standard manner. Thin sections were viewed with or without poststaining.

Cytochemical Localization of Cellulase (modified from Bal, 1972)

Substrate:

carboxymethyl cellulose (type 7 MF)	1.0 mg
0.018 M citrate buffer, pH 5.0	10.0 ml

Hyphae were fixed for 1 hr on ice and washed with cold buffer overnight. They were next transferred directly to substrate solution for 10 min to 2 hr at RT. After incubation, hyphae were transferred to 80 C Benedict's solution (Bauer et al., 1968) for 5 min, washed in distilled water, and postfixed in osmium tetroxide as described earlier. Subsequent treatment adhered to standard techniques.

Cytochemical Localization of Acid Phosphatase (Gomori, 1952)

Substrate:

Na-β-glycerophosphate	30.0 mg
0.05 M acetate buffer, pH 5.0	11.0 ml

12% w/v lead nitrate	0.1 ml
sucrose	0.8 g

Hyphae were fixed by the standard method; however, 7.5% sucrose was included in the fixation medium and in all buffer washes because hyphal tips tend to burst in dilute acidic solutions (Park and Robinson, 1966). After fixation, hyphae were washed in cacodylate buffer, followed by 0.05 M acetate buffer, pH 5.0, and incubated in substrate solution for 45 min at 37 C. Following a rinse in acetate buffer, samples were postfixed in OsO_4 and embedded as described previously. Thin sections were observed after poststaining with lead citrate.

ELECTRON MICROSCOPY OF HYPHAL APICES

It is the aim of this research to study the location of the enzyme cellulase (Cx) within hyphae of A. ambisexualis and to determine what evidence, if any, exists to implicate this enzyme in wall morphogenesis during vegetative growth. In this section, growing hyphae will be examined with emphasis being placed on the involvement of subcellular structures in wall formation.

The Cytoplasmic Organization of Achlya Hyphal Apices

Growing fungal hyphae extend at their tips, and this process is reflected in the organization of the apical region (Grove and Bracker, 1970). Hyphal apices of A. ambisexualis were examined electron microscopically, in order to compare the apical organization of this fungus with that of other fungi.

Methods

Fungal hyphae were obtained in either of two ways. In the first method, #2 cork borer plugs were removed from the edge of a 48 hr old Enriched Agar Medium colony and placed in a 5 cm petri plate which contained about a 5 mm deep layer of DLM. The culture was incubated at RT for 8-12 hr, after which time hyphae had grown out from the agar plugs to a distance of 2-5 mm. Agar plugs bearing hyphae were then processed for electron microscopy according to standard methods.

In the second method, a small piece of mycelium was transferred from a YPSS agar slant to a 5 cm petri plate containing about a 2 mm deep layer of Enriched Agar Medium, and the culture was incubated at RT for 12-24 hr. The entire layer of agar with submerged hyphae was then processed for electron microscopy, as previously described.

These two methods produced robust, straight hyphae, which are vastly more suitable for electron microscopy than are the narrow, contorted hyphae that are obtained from shaken liquid cultures. As a result, hyphae can be conveniently manipulated with minimal risk of damage, and embedded specimens can be easily oriented for longitudinal sectioning.

Observations

The apical regions of Achlya hyphae, like those of other fungi, can be divided into the extensive subapical zone, which contains an abundance of organelles and no central vacuole (Fig. 1), and the apex-proper, which is characterized by its population of small apical vesicles (Fig. 2). Walls in thin sections taken from both zones are not contrasted by poststaining with lead citrate (e.g., Figs. 1,6), but poststaining with $BaMnO_4$ reveals their presence (e.g., Figs. 2,8). While the width of the wall varies greatly in each region, measurements indicate a width of 178 ± 100 nm for older, lateral walls and a width of 59 ± 20 nm at the very apex. This agrees very well with measurements of the cell walls of Phytophthora hyphae, which are 175 nm wide laterally and 54 nm wide at the apex (Hunsley, 1973).

Organelles in the subapical zone include nuclei, mitochondria, endoplasmic reticulum (ER), dictyosomes (Golgi apparatus), and numerous cytoplasmic vesicles. These vesicles are of various sizes, shapes, and

Fig. 1. Longitudinal section of the subapical region of an A. ambisexualis hypha. M, mitochondrion; N, nucleus. x 5,500. All magnifications are approximate.

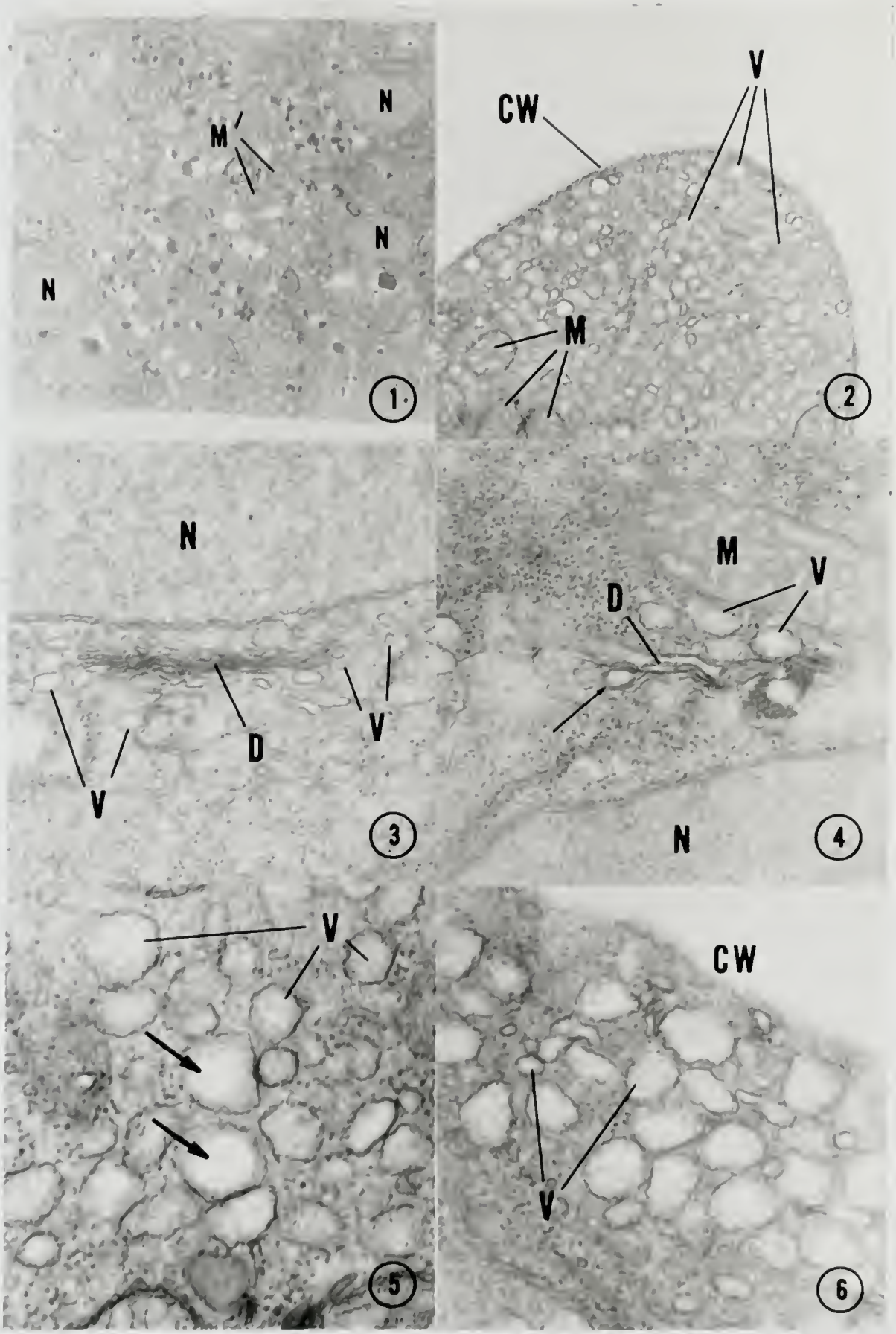
Fig. 2. Longitudinal section of the apical region of an A. ambisexualis hypha, which shows the many cytoplasmic vesicles (V) in the region. The secion was poststained with $BaMnO_4$ to reveal the cell wall (CW). M, mitochondrion. x 22,000.

Fig. 3. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows a dictyosome (D) surrounded by cytoplasmic vesicles (V). N, nucleus. x 48,000.

Fig. 4. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows a dictyosome (D) bearing an incipient fibrous vesicle (arrow). M, mitochondrion; N, nucleus; V, vesicle. x 43,000.

Fig. 5. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows details of the cytoplasmic vesicles (V). Arrows indicate the central fiber-free zone of two vesicles. x 90,000.

Fig. 6. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows cytoplasmic vesicles (V) beneath the cell wall (CW). The wall is not contrasted with the lead citrate poststain. x 75,000.


N
M
N
N
N
1
CW
V
M
2
N
V
D
V
3
M
V
D
N
4
V
5
CW
V
6

contents; and many are located near dictyosomes (Figs. 3,4,9). Indeed, dictyosomes bear what appear to be incipient vesicles and may be one source of the many cytoplasmic vesicles (Figs. 4,9). There is a virtual continuum of vesicle sizes, with diameters commonly extending from about 40 nm to about 160 nm. The largest of these vesicles (those with diameters above about 120 nm) exhibit a characteristic morphology in thin section, which consists of a fibrous matrix found mostly in the peripheral region of the vesicle's interior; the innermost region often appears free of fibrous material (Fig. 5).

In the subapical region, vesicles are commonly found just beneath the cell wall (Fig. 6). These "vesicles," however, are not always the roughly spherical structures implied by the term. Tangential sections of hyphae reveal that some of these structures are quite elongated and may be better described as submural tubules (Fig. 7). These correspond in morphology to the large vesicles with fibrous contents (Fig. 5). Most such structures, though, appear to be legitimate vesicles, and the possibility exists that submural vesicles and submural tubules are distinct, but related, structures. Perhaps, one gives rise to the other; structures intermediate between tubules and vesicles (Fig. 8) may represent fusion of vesicles or vesicle production. Some vesicles that are attached to dictyosomes (Fig. 4) have a peripheral fibrous matrix like that of large fibrous cytoplasmic vesicles, suggesting that this may be their true origin. But the resolution of questions like this is very difficult using thin-sectioned material.

The smaller vesicles (those with a diameter less than about 120 nm) may have contents which appear either fibrous or featureless (Figs. 5,6), but in most, the fibrous nature of the contents is difficult to discern.

Fig. 7. Tangential longitudinal section of the subapical region of an A. ambisexualis hypha. Arrows indicate elongated configurations of "submural tubules." The cell wall (CW) is not contrasted by the lead citrate poststain. x 24,000.

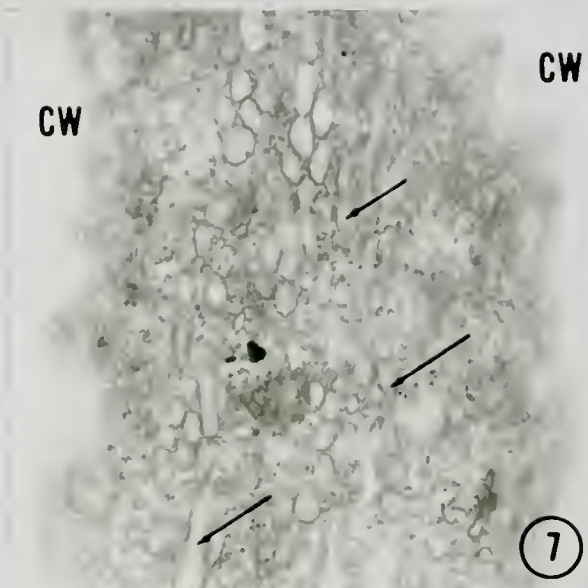
Fig. 8. Longitudinal section of the subapical region of an A. ambisexualis hypha. The arrow indicates a structure that may be intermediate between a tubule and vesicles. The cell wall (CW) is contrasted with $BaMnO_4$. M, mitochondrion. x 30,000

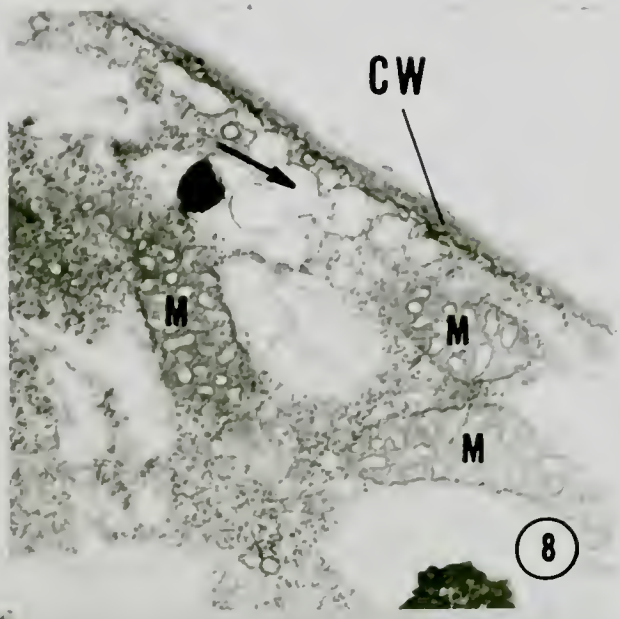
Fig. 9. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows coated vesicles (CV) in the vicinity of and attached to dictyosomes (D). M, mitochondrion; N, nucleus. x 48,000.

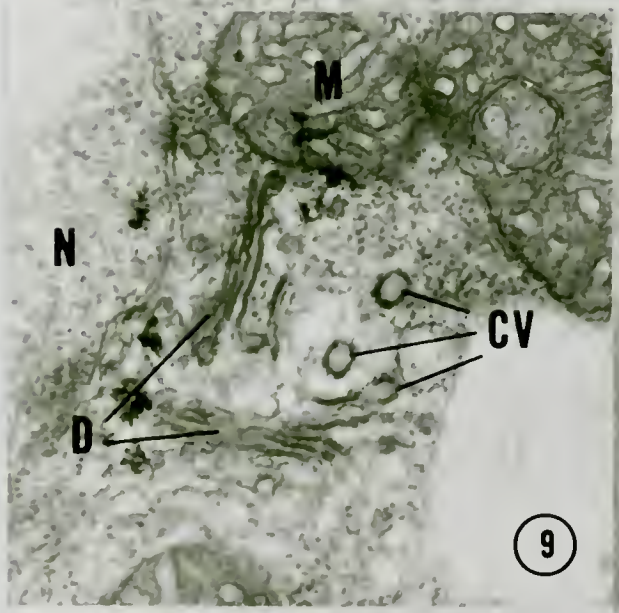
Fig. 10. Longitudinal section of the apical region of an A. ambisexualis hypha, which shows the lack of deposition of cellulase reaction product in the hypha. CW, cell wall; M, mitochondrion; V, vesicle. x 24,000.

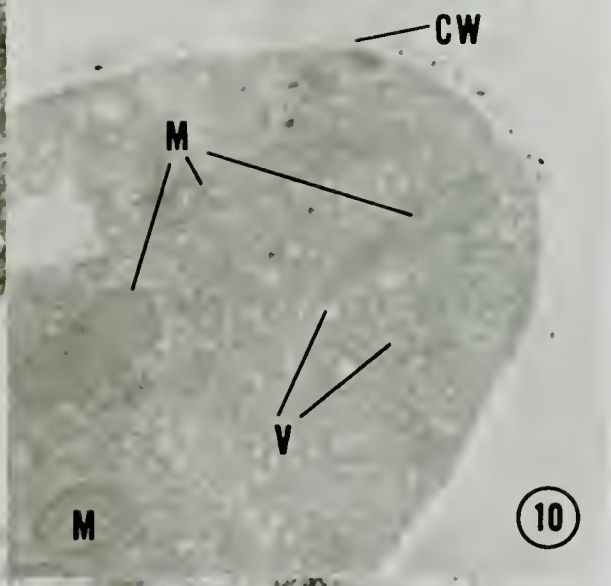
Fig. 11. Longitudinal section of the apical region of an A. ambisexualis hypha, which shows the distribution of acid phosphatase-positive and acid phosphatase-negative vesicles in the apex. CW, cell wall; M, mitochondrion; V. vesicle. x 22,000.

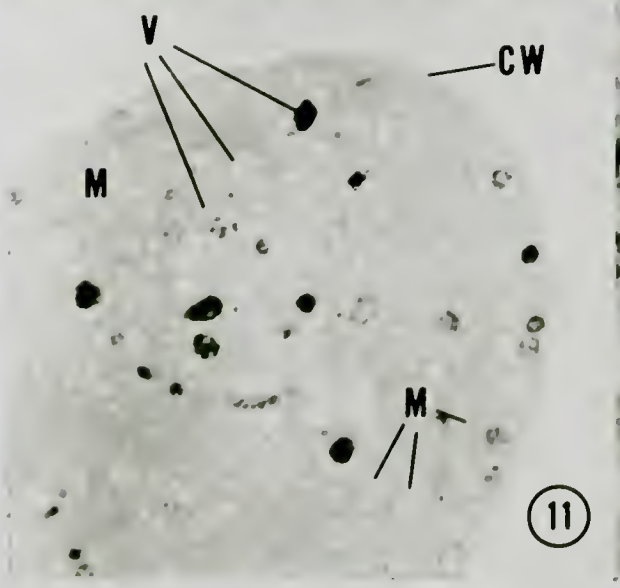
Fig. 12. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows the deposition of acid phosphatase reaction product in some of the cytoplasmic vesicles, but not in others. M, mitochondrion; V, vesicle. x 55,000.

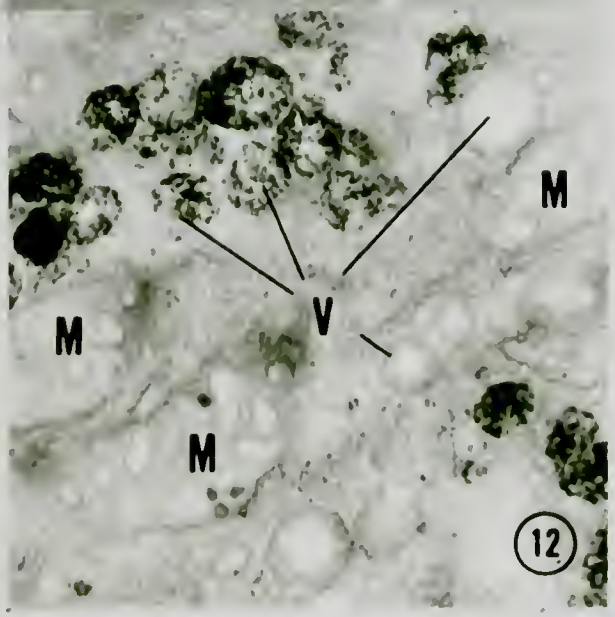

CW
CW
7


CW
M
M
M
8


M
N
CV
D
9


CW
M
V
M
10


V
CW
M
M
11


M
V
M
M
M
12

If the contents are fibrous, an inner "fiber-free" zone is usually not present. Another kind of vesicle that is present is the coated vesicle, which has a diameter of 85 ± 5 nm; this is apparently produced by dictyosomes (Fig. 9). These are the same size as the coated vesicles in the cytoplasm of radish root hairs (Bonnett and Newcomb, 1966).

The apical region is populated for the most part by vesicles, though mitochondria intrude almost to the apical wall itself (Fig. 2). These vesicles seem to be identical to the vesicles already described, though elongated profiles reminiscent of "submural tubules" are not commonly seen in the apex, and most apical vesicles would seem to be true spheres. No sections revealed images that could be interpreted as representing stages in the fusion between vesicles or the fusion between a vesicle and the plasma membrane.

Cytochemical Localization of Enzymes and Other Materials in Hyphal Apices

Methods

Cytochemical tests were performed using the techniques already described under Materials and Methods.

Results

Cellulase. The attempt to use this technique was apparently unsuccessful; no reaction product was observed in any part of the hyphae examined (Fig. 10).

Acid phosphatase. The reaction product of the acid phosphatase reaction is located in some 20-30% of the apical vesicles present in the terminal 3 μm of the apex (Fig. 11), and the average size of the reactive vesicles is 143 ± 19 nm. The proportion of reactive vesicles

in the overall vesicle population increases with distance from the apex. The reaction product is preferentially associated with the fibrous contents of those vesicles that stain, but nearby vesicles of similar morphology may not stain at all (Fig. 12).

Stain in dictysomes can also be seen (Fig. 13), and it is restricted to a single cisterna in those dictyosomes that react; reaction product is also found in nearby small vesicles.

IDPase. Like acid phosphatase, IDPase activity is found in association with the fibrous material of large cytoplasmic vesicles, which measure 142 ± 18 nm in diameter (Fig. 14). Similarly, not all such structures react; but unlike acid phosphatase, IDPase is found in a much higher proportion of apical vesicles (Fig. 15).

What may be an as yet unrecognized class of apical vesicles is represented by an intensely reactive vesicle in which the reaction product is deposited both within and without the membrane (Fig. 14). The outside diameter of these structures is 89 ± 16 nm. In size and the presence of material outside the vesicle membrane, these IDPase-positive vesicles resemble the dictyosome-derived coated vesicles (Fig. 9).

Dictyosomes are unreactive, as are attached incipient vesicles (Fig. 16).

Alkaline phosphatase. Alkaline phosphatase reaction product is not associated with any cellular structure, including dictyosomes (Fig. 17) and cytoplasmic vesicles (Fig. 18). The fine electron-dense deposits associated with the cytoplasmic vesicles and dictyosomes are also present in control material from which β-glycerophosphate was omitted

Fig. 13. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows the deposition of acid phosphatase reaction product in a single cisterna of a dictyosome (D). M, mitochondrion; N, nucleus. x 54,000.

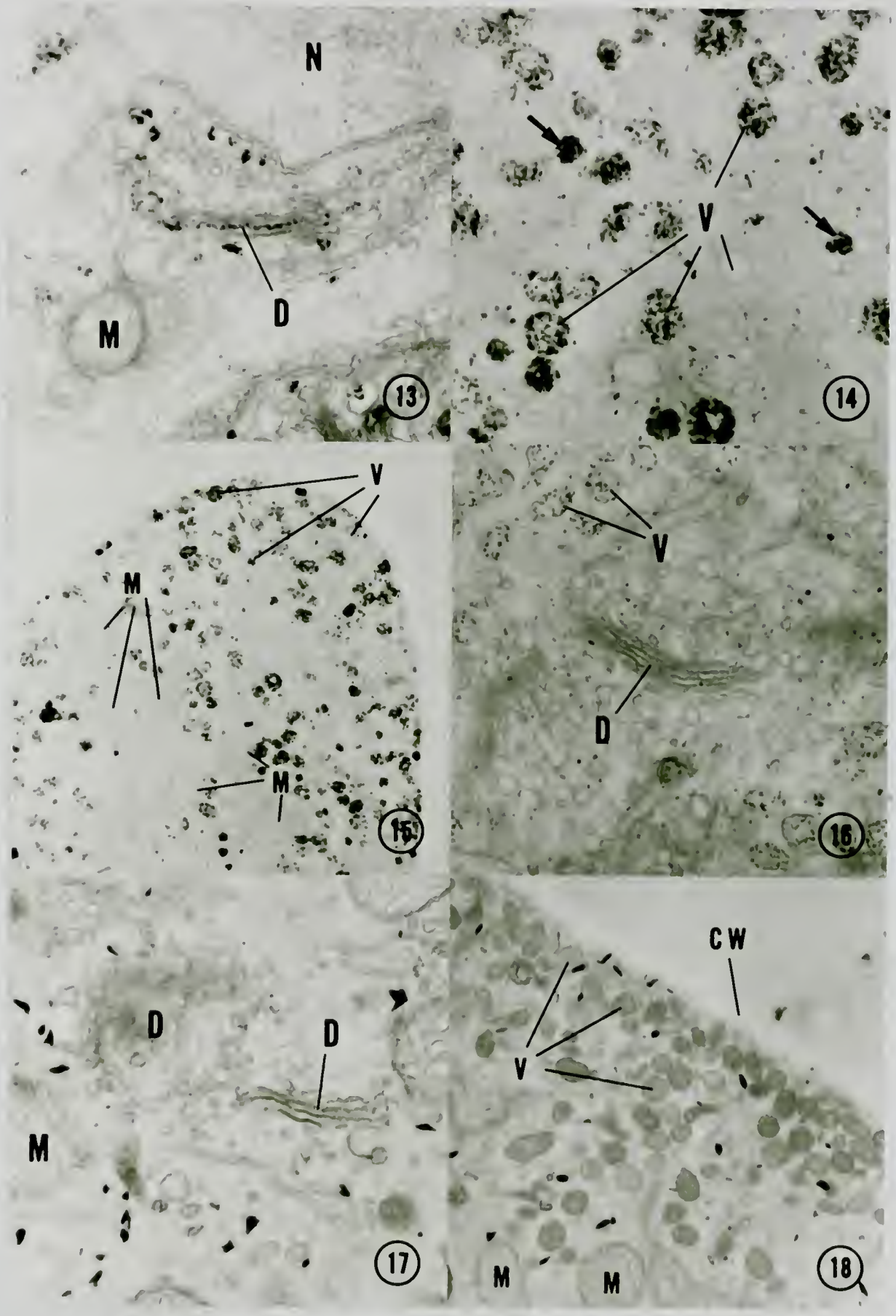
Fig. 14. Longitudinal section of the apical region of an A. ambisexualis hypha, which shows the deposition of IDPase reaction product in some of the cytoplasmic vesicles, but not in others. Arrows indicate smaller vesicles, in which the reaction product is deposited both inside and outside the membrane. V, vesicle. x 49,000.

Fig. 15. Longitudinal section of the apical region of an A. ambisexualis hypha, which shows the distribution of IDPase-positive and IDPase-negative vesicles in the apex. M, mitochondrion; V, vesicle. x 19,000.

Fig. 16. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows the lack of IDPase reaction product in a dictyosome (D). V, vesicle. x 43,000.

Fig. 17. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows the lack of alkaline phosphatase reaction product in dictyosomes (D). The finely granular deposit in dictyosomes and the coarse cytoplasmic deposit are nonspecific products, which are also present in control incubations. M, mitochondrion. x 45,000.

Fig. 18. Longitudinal section of the apical region of an A. ambisexualis hypha, which shows the lack of alkaline phosphatase reaction product in cytoplasmic vesicles (V). The finely granular deposit in vesicles and the coarse cytoplasmic deposit are nonspecific products, which are also present in control incubations. CW, cell wall; M, mitochondrion. x 25,000.


N
D
M
13
V
14
V
M
M
15
V
D
16
D
D
M
17
CW
V
M
M
18

(not shown), as are the coarser deposits that are scattered throughout the cytoplasm.

PTA-CrO_3 stain. Only two types of membranes stain with the PTA-CrO_3 stain: the plasma membrane (Fig. 19) and some of the cytoplasmic vesicles (Figs. 19, 20). These vesicles are 86 ± 18 nm in diameter; the membranes of the larger fibrous vesicles do not stain (Fig. 19). In some of the reactive vesicles, only part of the membrane is stained (Fig. 19), though in most, the staining is complete.

PASM stain. PASM, a cytochemical stain for polysaccharide, stains most cell structures to some degree and has the highest "background" reactivity of the techniques employed. This is also true of control sections from which periodate oxidation has been omitted (not shown). Sections oxidized by periodic acid show enhanced deposition of silver grains primarily in three cellular locations: the cell wall (Fig. 21), dictyosomes (Fig. 22), and fibrous cytoplasmic vesicles (Fig. 21). In addition, the plasma membrane may react, but if it does, the reaction is masked by the heavy silver deposition in the cell wall.

The size of the reactive cytoplasmic vesicles is 152 ± 24 nm (the same size as the large IDPase-positive and acid phosphatase-positive vesicles), and virtually all vesicles of this size react. The silver reaction product is preferentially deposited in the region of the fibrous matrix (Fig. 21), and the inner fiber-free zone is unstained.

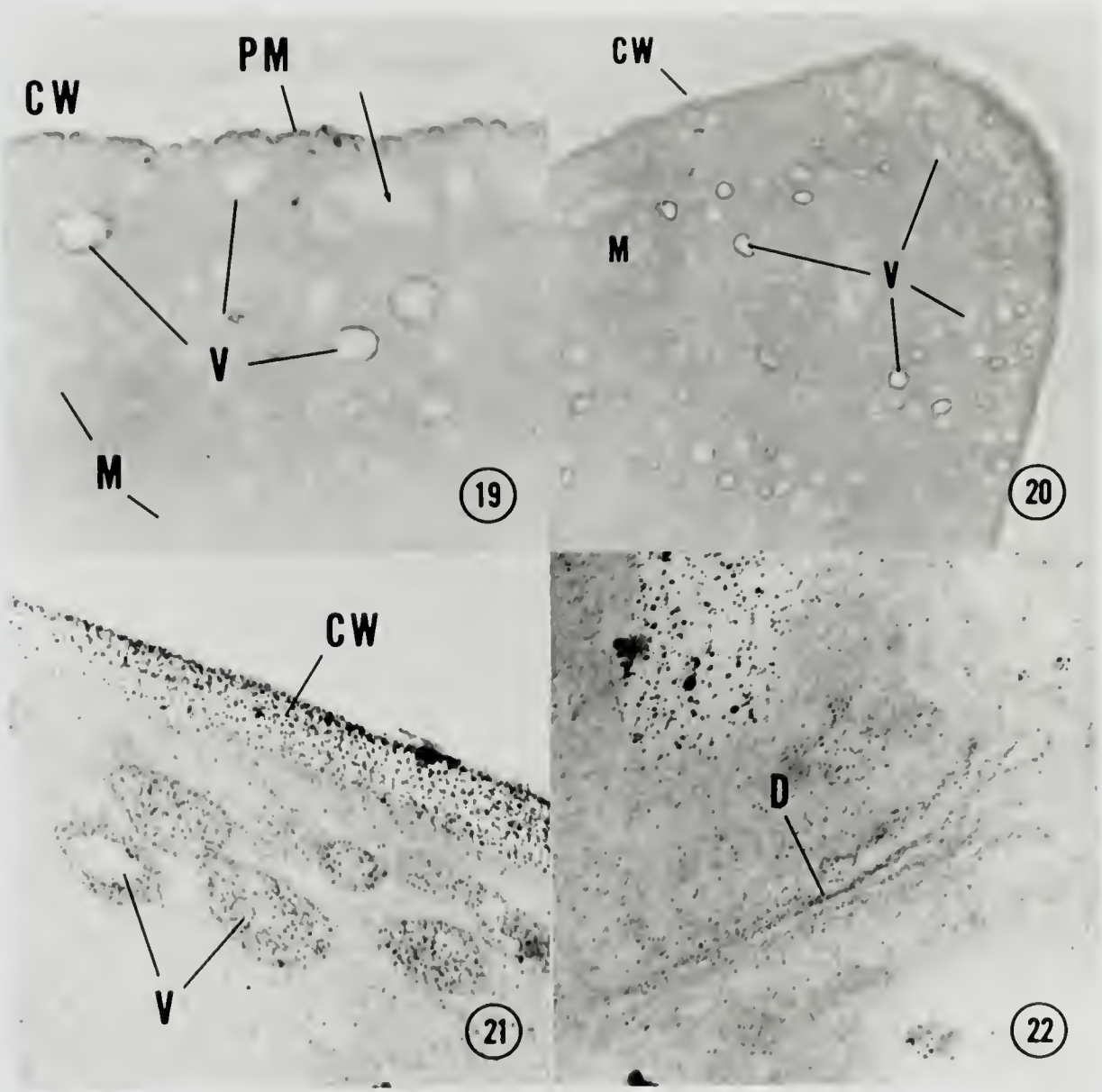
In many of the dictyosomes, cisternae at one pole are stained more intensely than cisternae at the opposite pole (Fig. 22).

Fig. 19. Longitudinal section of the apical region of an A. ambisexualis hypha, which shows the stainability of the plasma membrane (PM) and some (but not all) of the cytoplasmic vesicles with the PTA-CrO_3 stain. The arrow indicates a large fibrous vesicle. CW, cell wall; M, mitochondrion; V, vesicle. x 68,000.

Fig. 20. Longitudinal section of the apical region of an A. ambisexualis hypha, which shows the distribution of PTA-CrO_3-positive vesicles in the apex. CW, cell wall; M, mitochondrion. x 23,000.

Fig. 21. Longitudinal section of the apical region of an A. ambisexualis hypha, which shows the stainability of cytoplasmic vesicles (V) and the cell wall (CW) with the PASM stain for carbohydrate. x 79,000.

Fig. 22. Longitudinal section of the subapical region of an A. ambisexualis hypha, which shows a dictyosome (D) that has been stained with the PASM stain for carbohydrate. x 80,000.


CW
PM
V
M
19
CW
M
V
20
CW
V
21
D
22

Discussion

On the whole, the apical organization of *Achlya* hyphae agrees very well with the standard Oomycete pattern that was described by Grove and Bracker (1970). In particular, the apical zone is occupied largely by cytoplasmic vesicles, though the intrusion of mitochondria into this region may represent a slight deviation from the formal model. Another point of agreement is the morphological evidence that suggests that some or all of the cytoplasmic vesicles may be derived from the Golgi apparatus, and many of these vesicles contain polysaccharide. Polysaccharide has been detected cytochemically in cytoplasmic vesicles of several fungi, including *Achlya* (Dargent, 1975), *Phytophthora* (Meyer *et al.*, 1976), and *Saprolegnia* (Heath *et al.*, 1971) among the Oomycetes.

The stainability of dictyosomes with PASM is also common, and the fact that the more mature cisternae are the first sites of stainability in the endomembrane system is often taken as evidence that polysaccharide synthesis is initiated in this organelle (Pickett-Heaps, 1968).

From their morphological appearance and cytochemical behavior, it would appear that at least two types of apical (cytoplasmic) vesicles exist. The first consists of the large (about 150 nm) vesicles with PASM-positive fibrous matrices. These are also uniformly PTA-CrO_3-negative. Furthermore, most (but not all) vesicles of this type are IDPase-positive, while a smaller number contain acid phosphatase. It was not determined whether these two phosphatase activities are mutually exclusive or whether both can be found together in the same vesicles. If this latter case is true, then yet a third subclass must be recognized

in those 150 nm vesicles which contain neither activity. In either event, the strong probability exists that all 150 nm fibrous vesicles merely represent one or another stage of development, which is reflected in their variable enzyme content. If that is so, it is likely that acquisition of IDPase activity occurs as acid phosphatase activity is lost, because the dictyosomes are IDPase-negative and acid phosphatase-positive; and more of the vesicles in the apex exhibit activity of IDPase than of acid phosphatase.

Cytochemical localization of acid phosphatase in other fungi has demonstrated activity in dictyosomes; for example, Meyer et al. (1976) reported acid phosphatase activity in a single cisterna of Phytophthora dictyosomes. This is the same result observed in Achlya (Fig. 13). Acid phosphatase has traditionally been considered a lysosomal enzyme (Wattiaux, 1969); and it is found in various fungal vesicles, which may be lysosomes (Armentrout et al., 1976), though in many fungi it seems to be an enzyme of larger cellular vacuoles (Matile, 1971). Neither of these sites is comparable to the apical vesicles of Achlya; in fact, studies of fungal lysosomes generally report a lack of acid phosphatase activity in apical vesicles (Armentrout et al., 1976; Maxwell et al., 1978), though Dargent and Denisse (1976) report acid phosphatase activity in apical vesicles of Achyla bisexualis Coker.

An observation that may be of particular importance is that IDPase is not present in dictyosomes. This has been a traditional marker enzyme for dictyosomes, especially in plants (Dauwalder et al., 1972), although not all plant dictyosomes necessarily react (Dauwalder et al., 1969). In addition to its common association with plant dictyosomes, IDPase has been linked to polysaccharide synthesis (Ray et al., 1969).

The fact that IDPase is very specific for 150 nm polysaccharide-rich cytoplasmic vesicles may indicate a similar association with polysaccharide synthesis in Achyla. Additionally, this enzyme may serve as a marker for this class of apical vesicles in biochemical studies.

The second group of cytoplasmic vesicles consists of those smaller vesicles (about 80 nm), among which are found the PTA-CrO_3-positive vesicles, the small IDPase-positive vesicles, and the coated vesicles. These last two are probably identical, judging from their morphology; and the PTA-CrO_3-positive vesicles may also be identical. However, this has not been demonstrated, and the possibility must be accepted that this class of small vesicles is in fact composed of distinct subgroups.

The exact chemical basis for the PTA-CrO_3 reaction is unknown, but it is thought that glycoproteins that are characteristic of plasma membranes are involved (Roland et al., 1972). It is of interest, therefore, that certain cytoplasmic vesicles exhibit the same stainability as that shown by the plasma membrane. This has also been observed in higher plant cells, where it has been interpreted as the result of a chemical change in cytoplasmic vesicles that are involved in secretion (Vian and Roland, 1972). Presumably, as the vesicle membranes acquire the characteristics of the plasma membrane, certain barriers to their eventual fusion are overcome. In higher plants, the change is not uniform at first but is initiated in a restricted part of the vesicle; as a result, the membrane may display only partial stainability (Vian and Roland, 1972). Such is also true of cytoplasmic vesicles in Achlya. If this change is indicative of those vesicles which are capable of fusing with the plasma membrane, it is curious that none of the 150 nm fibrous

vesicles are PTA-CrO_3-positve. Can it be that these do not fuse with the plasma membrane?

It should be clear from the preceding discussions that at least two groups of vesicles can be found in the cytoplasm: the large fibrous vesicles and the smaller nonfibrous vesicles. In addition, each group may be composed of further forms which may be distinct or which may represent various stages in the development of the two major classes of vesicles. The existence of more than one type of vesicle in association with fungal wall formation has been frequently noted (Grove et al., 1970; Bracker, 1971; Hemmes and Bartnicki-Garcia, 1975; Meyer et al., 1976). Some investigators have concluded that the different vesicles may have different origins (e.g., from dictyosomes or from ER) and different functions (Meyer et al., 1976). Another possibility is that all vesicles are derived from the same source and that the larger vesicles are formed by the coalescence of the smaller ones (Grove et al., 1970).

In Achlya, no evidence was observed to justify the derivation of apical vesicles from the ER. Nor was there any evidence that 150 nm vesicles must be derived from the coalescence of vesicles of the 80 nm class. Instead, each class seems to have been independently produced by dictyosomes (Figs. 4,9). Though those fibrous vesicles which are still attached to dictyosomes (Fig. 4) are smaller than their mature size (ca. 90 nm vs ca. 150 nm), it is not necessary that their subsequent increase in size result from fusion with vesicles of the 80 nm class. Instead, the increase could result from the fusion of other small fibrous vesicles or from simple incorporation of materials.

The nature and function of the elongated "submural tubules" is not apparent. From their morphology, they would seem to be no more than

alternate forms of the large fibrous vesicles. As mentioned earlier, forms intermediate between the tubular and vesicular morphology may indicate that one is derived from the other (Fig. 8). Their location in the cell is well removed from the apex, where the bulk of incorporation of materials into the wall occurs. Instead, these tubules and vesicles are found as much as 20 or 30 μm behind the apex. A direct role in apical growth, thus, seems unlikely.

The cytochemical localizations of alkaline phosphatase and cellulase were unable to demonstrate activity in association with subcellular structures. Alkaline phosphatase has been reported to be associated with fungal ER and/or dictyosomes (Meyer et al., 1976) and with apical vesicles (Dargent, 1975). This latter report dealt with Achlya bisexualis, and it is surprising that no such association was found in A. ambisexualis apices. It must, of course, be acknowledged that this may merely be the result of an improper application of the technique in this study.

The greatest disappointment of this investigation must be the failure of the cytochemical test for cellulase activity, whose localization is the main object of the entire study. The failure is surprising, because the technique has already been employed successfully in another study of A. ambisexualis, strain E87 (Nolan and Bal, 1974). In that investigation, cellulase was reported to be localized in cytoplasmic vesicles (about 165 nm in diameter) of antheridiol-induced hyphae. These vesicles would appear to be identical to the 150 nm fibrous vesicles reported in this study, though the presence of cellulase in fibrous vesicles in noninduced mycelia cannot be automatically assumed.

Upon contemplation, it is interesting to note that the only published studies in which the cytochemical localization of cellulase has been successfully reported are those in which the technique has been performed by its originator, A. K. Bal (see Nolan and Bal, 1974; Bal et al., 1976 and references cited therein). While it would appear from these that the technique holds the promise of success, it may be that the published methodology for its successful application is incomplete.

CELLULASE AND UDPG TRANSFERASE

In this section, the existence of particle-bound cellulase (Cx) will be demonstrated, and some properties relevant to its bound state will be investigated. In addition, some features of UDPG transferase will be examined.

Some Properties of Mycelial Cellulase

The Distribution of Cellulases between Particulate and Soluble Fractions of Mycelial Homogenates

Methods

Mycelia were harvested from 48 hr old cultures and divided into lots weighing 2 g FW. These were homogenized with 2.0 ml of a homogenizing solution composed of 30% sucrose in 0.03 M tris·HCl buffer, pH 7.6, according to the standard scheme. Sand and cell fragments were removed by centrifuging the homogenate at 270 x g x 10 min, and a "total particulate" fraction was obtained by centrifuging the 270 x g supernatant at 37,000 x g x 90 min. This particulate fraction was resuspended in 2.0 ml of 10% sucrose in 0.01 M tris buffer, and 1.0 ml samples of the "particulate" fractions and of the 37,000 x g supernatant "soluble" fractions were assayed directly for cellulase activity.

As a comparison, another technique for extracting mycelial cellulase was employed, which has already been used in studies of Achlya (e.g., Thomas and Mullins, 1967). Two-gram lots of mycelium were

frozen on dry ice and ground with sand in a chilled mortar with 2.0 ml of 5.0% w/v NaCl. The slurry was centrifuged at 35,000 x g x 15 min, and the supernatant was decanted and saved. Five volumes of 95% ethanol were added to the supernatant, which was then recentrifuged at 35,000 x g x 15 min. The pellet was resuspended in 2.0 ml of distilled water and centrifuged at 27,000 x g x 15 min. The final supernatant was saved for assay of cellulase.

One unit of Cx activity is defined as that amount of enzyme activity which is sufficient to cause a 1.0% decrease in flow time of the substrate in 1.0 hr. This definition differs from that used in previous investigations in this laboratory (e.g., Thomas and Mullins, 1967), where a 10.0% decrease in flow time was considered to be 1.0 unit of activity.

Results are displayed in Table 3.

Results

As Table 3 indicates, cellulase can be recovered both from particulate and buffer-soluble fractions extracted by grinding in a buffered osmoticum; and the amount in each is about equal, as assayed by this technique. (However, as the following experiments will reveal, the level of particulate cellulase activity has been greatly underestimated by this assay.) Precipitation of cellulase from NaCl extracts yields cellulase levels about equal to either the buffer-soluble or particulate fraction alone, i.e., about one half of that in both combined.

TABLE 3. Cellulase activity in salt-soluble or buffer-soluble and buffer-insoluble fractions from homogenates of replicate 2g FW samples of A. ambisexualis mycelium produced by the method of Thomas (1966) or by grinding in a buffered osmoticum, respectively.

Sample number	Fraction	Cx activity (units/g FW)	Average activity
1	NaCl-soluble	5.2	4.9 ± 0.4
2		4.4	
3		5.1	
4	buffer-soluble	6.6	5.3 ± 1.2
5		5.7	
6		3.7	
4	buffer-insoluble	3.9	5.4 ± 1.3
5		7.0	
6		5.3	

The Effect of Triton X-100 on the Activity of Particulate and Soluble Cellulases from Mycelial Homogenates

Methods

Ten grams of mycelium were homogenized in 5.0 ml of homogenizing solution, as described in the previous experiment. The homogenate was divided into "total particulate" and "buffer-soluble" fractions by centrifugation, and the particulate material was resuspended in 20 ml of 10% sucrose in 0.01 M tris buffer. Separate aliquots of resuspended particles were made 0%, 0.25%, 0.5%, 1.0%, and 2.0% w/w in triton X-100, and aliquots of the buffer-soluble phase were made 0% and 1% in triton. Samples (1.0 ml) were assayed for cellulase activity. Initial mixing produced a high degree of aeration in those viscometer tubes that contained triton, and viscosity changes were erratic during the first 15 minutes. Therefore, readings were made at T = 15 min and at T = 15 min + 1 hour. The results are displayed in Table 4.

Results

Treatment of cellular particles with 0.5%, 1.0%, or 2.0% triton X-100 increases cellulase activity on the average to about 8.7 times the activity of untreated particles. Triton at 0.25% gives a lesser degree of activation. Triton is not itself cellulolytic, as the comparison between the blanks with and without triton reveals. Finally, the activity of triton would seem not to involve an activation of the enzyme itself, but rather a specific effect upon the bound state is indicated. One possible mode of action is the freeing or solubilization of the enzymes from the particles to which they are bound. To determine whether this is involved in cellulase activation, a second experiment was performed.

TABLE 4. The effect of triton X-100 on the activity of particulate and buffer-soluble cellulases from A. ambisexualis mycelial homogenates

Sample	Percent triton X-100	Cellulase* activity
10% sucrose in tris	0.0	0.00 units
	2.0	0.02 "
Particulate fraction	0.0	5.41 units/g FW
	0.25	37.11 "
	0.5	46.18 "
	1.0	47.43 "
	2.0	45.88 "
Buffer-soluble fraction	0.0	4.18 units/g FW
	1.0	4.52 "

*Each value is the average of two measurements.

The Effect of Triton X-100 on Solubilization of Particle-bound Cellulase

Methods

Cellular particles were obtained and resuspended in 10% sucrose in tris buffer, as described in the preceding experiment. Separate aliquots of resuspended cellular particles were made 0%, 0.01%, 0.1%, and 1.0% w/w in triton X-100. Tritonated samples were kept on ice for 15 min to prevent complete triton activation and then centrifuged for 60 min in a 65 rotor at 79,000 x g. Sedimentable material was resuspended in 10% sucrose in tris buffer, and an aliquot was made 1% in triton X-100 at room temperature in order to activate any cellulase still bound to the particles. Cellulase activities of the 79,000 x g sediment and of the 79,000 x g supernatant (solubilized activity) were determined, and the results are displayed in Table 5.

TABLE 5. The distribution of Cx activity between particulate and soluble phases after treatment of A. ambisexualis cellular particles with various concentrations of triton X-100

Percent triton X-100	Cx activity bound to cell particles (units/g FW)*	Cx activity solubilized (units/g FW)*	Percent of total activity solubilized
0.0	67.7	4.1	6
0.01	79.4	9.2	10
0.1	41.4	34.7	46
1.0	13.9	69.3	83

*Each value is the average of two measurements.

Results

From Table 5, it can be concluded that bound cellulase exists in a relatively inactive form that can be activated by triton X-100, and this activation is accompanied by removal of cellulase from the particles. This type of behavior is typical of biological membranes.

The Effect of Various Methods for Solubilizing Membrane-bound Cellulase

Membrane-bound proteins have been divided into two classes: integral and peripheral (Singer, 1974). Their distinction rests upon the tenacity with which each is bound to the membrane and upon the types of treatments required for their removal or solubilization. To better determine the strength with which cellulase is bound to cellular membranes, the following experiments were performed, in which salts, sonication, and freezing were used in an attempt to remove cellulase from cellular membranes.

Methods

Cellular particles were obtained and resuspended in 10% sucrose in tris buffer, as described previously. Separate 1.0 ml aliquots were subjected to one of the following treatments, which are designed to remove trapped or lightly bound cellulase: sonication twice for 30 sec each at setting #5 of a Heat Systems-Ultrasonics sonifier-cell disruptor, model W185, fitted with a standard microtip (samples were kept chilled); freeze-thawing twice at -70 C; making solutions 1.0 M in NaCl, KCl, or NH_4Cl for 15 min at RT. Following these treatments, samples were centrifuged for 60 min at 79,000 x g, and the sedimentable materials were resuspended in 10% sucrose in tris buffer. Aliquots of resuspended 79,000 x g sediments were made 1.0% in triton X-100 to activate bound

cellulase, and both 79,000 x g sediment and 79,000 x g supernatant fractions were assayed for Cx activity and protein content. Results are displayed in Table 6.

Results

Freeze-thawing and monovalent cations are ineffective in dislodging cellulase from membranes. The salts used are quite effective in removing proteins, however (about a 25% increase in soluble protein), so cellulase must be more tightly bound than at least 25% of the other membrane proteins. While sonication results in the loss of some 10% of the cellulase from the particulate fraction, this is accompanied by the loss of a comparable amount of total protein, and may, therefore, represent membrane fragmentation, rather than solubilization. In any event, the release of cellulase by sonication is not of the same magnitude as the release achieved by triton treatment (Tables 4 and 5).

The Effect of Room Temperature Incubation on Activity of Membrane-bound Cellulase

During the course of these experiments, it was observed that on some, but not all, occasions, samples of resuspended cellular particles that had been left "unattended" at room temperature for a number of hours displayed unexpectedly high cellulase activity. Much further investigation revealed that these "activated" samples were among those obtained from mycelium which had been homogenized in a medium containing dithiothreitol (DTT). Though the membranes had been resuspended in media lacking DTT, it was possible that minute amounts were carried over in some samples and were somehow affecting enzyme activity. Experiments were conducted to determine the effect of DTT and room temperature "aging" on the activity of membrane-bound cellulase.

TABLE 6. The distribution of protein and Cx activity between particulate and soluble phases after treatment of A. ambisexualis cellular membranes with salts, freezing, or sonication

Treatment	Cx activity bound to cell particles (units/g FW)*	Soluble Cx activity (units/g FW)*	Percent soluble Cx activity	Protein bound to cell particles (mg/g FW)*	Soluble protein (mg/g FW)*	Percent soluble protein
None	67.0	2.2	3.2	1.17	0.12	9.3
Freeze/thaw	67.0	2.0	2.9	1.17	0.13	10.0
Sonication	63.3	9.7	13.3	1.07	0.26	19.5
1 M NaCl	64.5	1.3	2.0	0.87	0.46	34.6
1 M KCl	69.9	0.8	1.1	0.87	0.44	33.6
1 M NH_4Cl	68.4	0.7	1.0	0.88	0.46	34.3

*Each value is the average of two measurements.

Methods

Ten grams of mycelium were homogenized, as previously described, and the total particulate fraction was resuspended in 20 ml of distilled water. Samples were made 0 mM, 0.5 mM, and 5.0 mM in DTT and incubated at room temperature. Periodically, 1.0 ml aliquots were removed and assayed for cellulase activity, without triton activation. Results are displayed in Table 7.

TABLE 7. The effect of DTT upon cellulase activity during incubation of A. ambisexualis cellular membranes for 24 hr at room temperature

Concentration of DTT	Cx activity* at T = 0 hr (units/g FW)	Cx activity* at T = 24 hr (units/g FW)
0.0 mM	4.9	11.3
0.5 mM	6.0	45.4
5.0 mM	7.9	45.4

*Each value is the average of two measurements.

Results

In the absence of DTT, cellulase activity increases about 2.3 times, whereas in the presence of DTT, activity increases by about 6 to 8 times the original value. This confirms the suspicion that the activity of membrane-bound cellulase can increase at RT and that DTT enhances the process.

The Effect of Temperature on Cellulase Activity during Incubation of Cytoplasmic Particles

Methods

Membrane samples were obtained as previously described and resuspended in distilled water made 0.5 mM in DTT. Portions were incubated at 4 C, 25 C (RT), and 37 C, and aliquots were removed at T = 0 hr and T = 24 hr for assay of cellulase activity without triton activation. Samples were also assayed for protein content. Results are displayed in Table 8.

TABLE 8. The effect of temperature upon cellulase activity during incubation of A. ambisexualis cellular membranes for 24 hr in 0.5 mM DTT

Temperature	Cx activity (units/g FW)†		mg protein/g FW†	
	T = 0 hr*	T = 24 hr	T = 0 hr*	T = 24 hr
4 C	5.1	9.0	2.43	2.48
RT	5.1	42.9	2.43	1.76
37 C	5.1	9.0	2.43	1.44

*Since all three T = 0 samples are identical, only one representative value was obtained.

†Each value is the average of two measurements.

Results

Cellulase activity increased 8.5-fold in 24 hours at RT, as expected. Activation is prevented at temperatures of 4 C and 37 C. One explanation may be that activation requires a temperature sensitive step (enzymatic?) with an optimum around 25 C. This may be proteolytic,

since protein content during "aging" decreases at room temperature, while no proteolysis is detected at 4 C. However, the greatest proteolysis occurs at 37 C, which shows no activation. Thus, proteolysis and activation may not be related. An alternative explanation might require that mild or selective proteolysis may occur at RT, causing activation, but that extensive proteolysis occurs at 37 C, and cellulase is degraded.

The Effect of Incubation at Different Temperatures on the Solubilization of Membrane-bound Cellulase

Activation of membrane-bound cellulase by triton has already been shown to be accompanied by solubilization of the enzyme (Table 5). However, a requirement of solubilization for activation was not demonstrated. To determine whether activation of cellulase by an apparently unrelated method also is accompanied by solubilization, the following experiment was performed with "aged" material.

Methods

Membrane samples resuspended in distilled water were obtained by the methods used in the preceding experiments. Samples of cellular particles, made 0.05 mM in DTT, were incubated at 4 C, RT, and 37 C for 24 hr, and aliquots were removed and centrifuged at 79,000 x g x 60 min. The resulting sediment and supernatant fractions were assayed for Cx activity directly, and a sample of the sediment fraction was also assayed in the presence of 1.0% triton X-100 to activate bound cellulase. Results are displayed in Table 9.

Results

As expected, directly assayable cellulase activity (i.e., the sum of soluble and particulate activities without added triton) is higher

TABLE 9. The effect of temperature upon solubilization of cellulase from A. ambisexualis cellular membranes during incubation in the presence of 0.5 mM DTT for 24 hr

Temperature	Cellulase activity (units/g FW)				
	Soluble activity*	Insoluble activity without 1% triton X-100*	Sum of soluble and insoluble-minus-triton activities	Insoluble activity plus 1% triton X-100*	Sum of soluble and triton-activated insoluble activities
4 C	3.7	3.2	7.1	90.9	94.6
RT	21.4	3.6	25.0	76.5	97.9
37 C	4.3	2.1	6.4	16.3	20.6

*Each value is the average of two measurements.

after room temperature incubation than after incubation at 4 C or 37 C. The bulk of this increase is clearly in the soluble phase. Compared to the material which has been incubated at 4 C, a smaller amount of triton-releasable cellulase activity is still associated with the membranes after RT incubation. The simplest explanation is that, with room temperature "aging," inactive membrane-bound cellulase becomes both active and soluble, and this process is retarded by low temperature. As evidence, notice that the maximum available cellulase activity in the 4 C and RT samples is approximately equal (94.6 vs 97.9), indicating that one effect of room temperature incubation is to shift Cx from the particulate phase to the soluble. The fact that less membrane-bound, triton-releasable cellulase remains after incubation at RT than after incubation at 4 C also indicates that both triton solubilization and "aging" act on the same pool of membrane-bound cellulase. Finally, this comparison between triton activation and room temperature activation suggests strongly that solubilization is a requirement for maximal Cx activity and is not just a secondary effect of activation.

The distribution of activities at 37 C, however, holds an unexpected result, because the triton-activatable particulate component is missing. Thus, unlike the lack of activation observed at 4 C, the lack of activation at 37 C is not merely due to inhibition of the room temperature effect by unfavorable temperature. That is, the membrane-bound cellulase activity has not been left in place, as it would have been at 4 C.

A number of possible explanations come to mind, not all of which were treated by experimentation. It may be that cellulase is denatured by a temperature of 37 C. This is not likely, since a hallmark of

fungal cellulases is their remarkable temperature stability (Whitaker, 1963, 1971). In addition, Thomas (1966) demonstrated that salt-extractable cellulases from Achlya exhibit a considerable degree of stability even at 100 C.

Another possibility is that temperature may exert some effect on the membrane itself, rendering the bound cellulase somehow unavailable, though not actually denaturing it. Or it may be that the cellulase actually is solubilized at 37 C, but that it is destroyed (perhaps by proteolysis) after solubilization. Extensive proteolysis has already been shown to occur at 37 C (Table 8).

To determine whether a cellulase from Achlya would be denatured at 37 C, samples of buffer-soluble cellulase were incubated at room temperature and at 37 C for 24 hr, and cellulase activity was measured. From an initial value of 4.53 ± 0.21 units/g FW at T_0, activity changed to 4.92 ± 0.08 units/g FW after 24 hr at room temperature and to 4.31 ± 0.01 units/g FW after 24 hr at 37 C.

Buffer-soluble cellulase, then, like other fungal cellulases, is not affected by 37 C to a degree sufficient to dismiss 37 C inactivation of particulate cellulase as mere denaturation. This explanation cannot be rigidly excluded, however, because the temperature stabilities of buffer-soluble and membrane-derived cellulases have not been compared. Thus, though there is reason to doubt an effect of 37 C directly on cellulase, the reason for the apparent destruction of membrane-bound cellulase activity is not known.

Some Properties of UDPG Transferase

The Distribution of UDPG Transferase Activity between Protoplasm and Wall Fractions of Mycelial Homogenates

UDPG transferases, if indeed they are involved in cell wall metabolism, would be expected to be located at points where wall components are assembled. Evidence already cited from other workers has indicated that the wall may be assembled in part within the protoplast and in part outside the plasma membrane. Accordingly, UDPG transferase activity was looked for in "wall" and "protoplasm" fractions of disrupted Achlya mycelium.

Methods

Two-gram FW samples of mycelium were harvested from a 48 hr old liquid culture and transferred to 15 ml conical glass centrifuge tubes. Four milliliters of homogenizing solution, consisting of 20% sucrose, 10 mM DTT, and 0.02 M tris·HCl buffer, pH 7.6, were added to each tube, and the tubes were chilled in a salted ice bath. When the temperature reached 0 C, the contents were sonicated for 15 sec on the Heat Systems-Ultrasonics sonifier at a setting of 5. The temperature, which had reached 5-10 C during sonication, was reduced by rechilling, and the contents were sonicated for another 15 sec. Fragmented hyphae were sedimented by centrifugation at about 1,000 x g x 10 min, and the supernatants were decanted and saved. Fragments were resuspended in 2.0 ml of half-strength homogenizing solution, sonicated, and centrifuged as before. This procedure was followed twice more, and the four 1,000 x g supernatants from each sample were pooled.

Wall fragments were resuspended in 2.0 ml of half-strength homogenizing solution, and a sample was examined under the phase contrast

microscope, where little cytoplasmic contamination was evident. UDPG transferase activity was assayed using samples of both wall and supernatant fractions, and protein content was determined. Results are displayed in Table 10.

TABLE 10. The distribution of protein and UDPG transferase activity between "wall" and "protoplasm" fractions of A. ambisexualis mycelial homogenates produced by sonication

Fraction	UDPG transferase activity*†	Protein content (mg/g FW)†	Specific activity**
Wall	1.53 ± 0.09	0.59 ± 0.02	2593
Protoplasm	2.12 ± 0.13	3.70 ± 0.06	573
Σ	3.65	4.29	851

*UDPG transferase activity is expressed as nmoles of glucose incorporated per min per g FW.

†Each value is the average of 3-4 measurements.

**Specific activity is expressed as pmoles of glucose incorporated per min per mg protein.

Results

About 42% of the total mycelial transferase activity is found in the wall fraction, whereas only about 14% of the protein assayed is found here. Accordingly, the specific activity of wall-bound transferase is almost five times higher than that in the protoplasm. It cannot be said with certainty that the high activity in the walls results from a large number of enzyme molecules in the wall, because the enzyme assay does not present transferase enzymes with exogenous glucose-acceptors but relies on their demonstrated ability to transfer glucose to endogenous

acceptors. High activity of wall transferases might be due to the nature of the acceptor available (i.e., wall), which may represent a more "ideal" acceptor than that available to cytoplasmic samples. Be that as it may, it is apparent that transferases are found both in cell walls and in cell protoplasm.

The Distribution of UDPG Transferase between Particulate and Soluble Fractions of Mycelial Homogenates

Methods

Mycelium was harvested from 48 hr old cultures and divided into lots weighing 2 g FW. These were homogenized by grinding according to the standard scheme in 2.0 ml of homogenizing solution consisting of 30% sucrose, 15 mM DTT, and 0.03 M tris·HCl buffer, pH 7.6. The "total particulate" fraction was obtained and resuspended in 2.0 ml of one-third-strength homogenizing solution; and 100 µl fractions of the 37,000 x g supernatant "soluble" fraction and the resuspended "particulate" fraction were assayed for UDPG transferase activity and for protein content. The results are displayed in Table 11.

Results

Comparing the results in Table 10 with those in Table 11, it can be seen that grinding in a mortar yields about 38% less cytoplasmic protein than does sonication (3.70 vs 2.30 mg/g FW), though the overall specific activities of the cytoplasmic fractions in Tables 10 and 11 are almost identical (573 vs 523 nmoles·min^{-1}·mg^{-1}). The particulate fraction holds nine times the transferase activity at about eleven times the specific activity of that in the soluble phase. The ratio of specific activities in wall, particulate, and soluble phases is 28:11:1.

TABLE 11. The distribution of protein and UDPG transferase activity between particulate and soluble protoplasmic fractions of A. ambisexualis mycelial homogenates produced by grinding with a mortar and pestle

Fraction	UDPG transferase activity*†	Protein content (mg/g FW)†	Specific activity**
Particulate	1.08 ± 0.02	1.03 ± 0.06	1050
Soluble	0.12 ± 0.01	1.27 ± 0.09	93
Σ	1.20	2.30	523

*UDPG transferase activity is expressed as nmoles of glucose incorporated per min per g FW.

†Each value is the average of 3-4 measurements.

**Specific activity is expressed as pmoles of glucose incorporated per min per mg protein.

Although no specific experiment was performed for this purpose, a comparison of the results of Tables 10 and 11 can serve as a "mixing" experiment to reveal the presence or absence of soluble cytoplasmic inhibitors of transferase activity. In Table 10, the specific activity of the cytoplasmic fraction is calculated from the protein content and enzyme activity of the combined particulate and soluble phases. In Table 11, the same value is calculated from separate assays of those two fractions. The two values differ by only about 9%, a discrepancy too slight to require the involvement of activators or inhibitors of transferase.

The Solubility of Radioactive Products of UDPG Transferase Activity

Biochemical characterization of the products of the UDPG transferase reaction was not attempted in this research. However, their solubility in various solvents was investigated to enable some comparison to be made with UDPG transferases reported from other systems.

Methods

Mycelia were homogenized, as in the preceding experiment. The "total particulate" fraction was resuspended in 2.0 ml of 0.01 M tris·HCl buffer with 5 mM DTT, and transferase activity was assayed using three times the volumes of reagents and sample normally employed. Ethanol-insoluble products were fractionated by the following procedure, which is a modification of that used by Van Der Woude et al. (1974):

1. Two washes with 2:1 v/v chloroform:methanol (combine washes)
2. Two washes with 85 C distilled water (combine washes)
3. Two washes with 85 C 1.0 N NaOH (combine washes)
4. One wash with RT water (combine with alkali washes)
5. Two washes with RT water (discard washes)

Each wash was terminated by centrifugation at about 1,000 x g x 5 min in a conical 15 ml centrifuge tube. The chloroform:methanol washes were evaporated, and the residue was resuspended in distilled water and transferred to a scintillation vial. Alkali washes were neutralized with HCl, and samples of these and the hot H_2O washes were transferred to separate scintillation vials. Finally, the alkali-insoluble residue was transferred in distilled water to a scintillation vial, and all samples were counted by standard procedures. The percent of radioactivity in each extract is displayed in Table 12.

TABLE 12. The distribution of radioactivity among different extracts of the products of UDPG transferase activity from the particulate fraction of A. ambisexualis mycelial homogenates

Extraction	Percent of radioactivity*
Chloroform/methanol	4.9 ± 2.5
Hot H_2O	27.1 ± 2.1
Hot NaOH	56.8 ± 3.4
Insoluble residue	11.3 ± 2.9

*Each value is the average of 3-4 measurements.

Results

The fractionation scheme employed is derived from higher plant cell wall methodology, where walls can be similarly fractionated to yield the chloroform/methanol-soluble wall lipids and glycolipids, the hot-water-soluble pectins and calloses, the hot-alkali-soluble hemicelluloses, and the alkali-insoluble α-cellulose (Siegel, 1968; Preston, 1974). Also, it is assumed that the products of transferases can be fractionated into similar classes of compounds. The wisdom of this assumption will be discussed later. At this point it will only be mentioned that, if the above assumption is taken literally, only about 10% of the products would qualify as cellulose on the basis of solubility; this is, perhaps only coincidentally, about equal to the proportion of cellulose in Achlya cell walls (Parker et al., 1963).

Discussion

Cellulase

Mycelial cellulase in *Achlya* is shown to exist in at least two pools after homogenization: one which is associated with cellular particles and another which is soluble. Attempts to dislodge particle-bound cellulase by further physical disruption (Table 6) are unsuccessful, and this indicates that the soluble cellulase is not derived from the same population of molecules as those in the particle-bound pool. That is not to say that the soluble cellulase may not originally have been associated with cellular particles before homogenization. Any number of factors during homogenization and isolation can lead to partial or total solubilization of enzymes (Lips, 1975). Byrne *et al.* (1975) reported a buffer-soluble cellulase in pea epicotyls, which eventually proved to have been released from the endoplasmic reticulum (Bal *et al.*, 1976). The buffer-soluble cellulase of *Achlya* may also have been associated with cellular particles before disruption, but the fact that it cannot be added to by further disruption of particles signifies a qualitative, not quantitative, distinction between the two pools. However, the difference may merely reflect different cellular locations of otherwise identical molecules and not necessarily the existence of distinct isozymes.

In previous work on *Achlya*, mycelial cellulase was reported as that activity which could be extracted from frozen mycelium by grinding in a salt solution (Thomas, 1966). While salt extraction of cellulases is a standard method in many systems (e.g., Lewis *et al.*, 1970), salt extraction of whole *Achlya* mycelium yields less cellulase than is made available by homogenization in buffer (Table 3). When one considers that

the latent, particle-bound cellulase in Table 3 has been underestimated perhaps by a factor of 10, the proportion of mycelial cellulase recovered from the salt-soluble fraction becomes even less significant. The amount of cellulase in the salt extract is about equal to the buffer-soluble pool, and this may be the only source of salt-soluble cellulase. Particle-bound cellulases probably do not contribute to the salt-soluble fraction, since neither freezing nor salts dislodges cellulase from the particles (Table 6). A contribution from wall-bound pools, as is seen in the cases of peas (Bal et al., 1976) and beans (Reid et al., 1974), cannot be ruled out, but the existence of similar wall-bound cellulases in Achlya was not investigated in this research.

Whereas salts and physical disruption are ineffective in solubilizing particle-bound cellulase, solubilization can be accomplished by triton X-100 (Table 5). Detergents act by disrupting cellular membranes (Singer, 1974), and the effectiveness of triton in this instance is evidence that the particles in question are cellular membranes. They will be considered as such henceforth. Some information on the degree of binding of cellulase to membranes is revealed in these solubilization experiments. The failure of strictly physical disruption (freezing and sonication) to release cellulase from membranes indicates that the enzyme is not merely trapped inside of a vesicle as an otherwise soluble molecule. Such a molecule would be expected to be released during the membrane breakage which would accompany these treatments. Thus the efficacy of triton cannot be limited to the mere rupture of membranes but must lie in its ability to completely disrupt and disperse the lipoidal membrane components.

The failure of salts to release cellulase indicates that the enzymes are not loosely bound to the membrane by weak, noncovalent bonds and are not peripheral proteins (Singer, 1974). That peripheral proteins of other types are present is attested to by the ~25% increase in soluble protein following salt treatment (Table 6). Bound cellulase, then, would seem to fall into the class of membrane proteins termed "integral" (Singer, 1974), i.e., they are present in the hydrophobic interior of the membrane, or they are strongly bound to such proteins.

The degree of binding exhibited by membrane-bound cellulase in Achlya has its counterpart in the membrane-bound cellulase of kidney bean abscission zones (Koehler et al., 1976). This, too, is an integral protein, which is released primarily by detergent and only to a much smaller degree by salts or physical disruption. A similar enzyme may be the β-glucanase in ER vesicles of yeast, which is activated by triton X-100 (Cortat et al., 1972).

Fungal cellulases are known for their diversity (Pettersson, 1963; Wood, 1968), and intracellular cellulases of individual higher plants may differ markedly in their substrate specificity, extractability, and molecular weight (Byrne et al., 1975). There is, therefore, no absolute requirement that the secreted, the buffer-soluble, and the membrane-bound cellulases of Achlya all be identical. However, in pea epicotyls, a comparable trio exists in the buffer-soluble, the particulate, and the wall-bound cellulases (Bal et al., 1976). Though at least two isozymes are involved, the evidence indicates that these are sequentially modified forms of the same enzyme (Wong et al., 1977b). Thus, the possibility exists that in Achlya, too, the two internal pools of

cellulase represent the same enzyme in different stages of production and secretion.

The strength with which cellulase is bound to cell membranes raises serious questions about the way in which it is secreted or whether membrane-bound cellulases can be secreted at all. If they are, at some point they must become soluble. Clearly, they are not already so, and their integral nature seems to argue against a mechanism for their quick release. It is, therefore, of considerable interest that membrane-bound cellulase can become soluble under conditions of adequate temperature and concentration of DTT. Although the mechanism whereby this occurs is not known, it seems to indicate that a strong degree of binding to cytoplasmic membranes need not be a barrier to an enzyme's eventual secretion.

The requirement, of course, is that cellulase release by "aging" be reflective of a natural cellular process and not a completely artificial phenomenon, such as gross degradation of membranes. A parallel may exist in a report by Frantz *et al.* (1973) in which the stainability of isolated dictyosome vesicles by PTA-CrO_3 was seen to increase with time after isolation. This change is also part of the natural maturation of the vesicles in the intact cell and apparently results from changes of these membranes toward a more "plasmalemma-like" state (Vian and Roland, 1972). Thus, changes in membranes *in vitro* can reflect the continuation of natural developmental processes. Room temperature activation of membrane-bound cellulase may be one such instance.

The requirement of DTT for enzyme release by "aging" raises the possibility that some protein is critical to the process. DTT protects protein sulfhydryl groups from oxidation (Cleland, 1964), thereby

preserving tertiary structure and/or the specificity of critical sites. In this way, DTT may enable cellulase release to occur through the action of a second enzyme, which may, for instance, be a protease. An instance has been reported wherein an integral membrane protein can be released in an active state by the activity of lysosomal enzymes; otherwise, triton treatment is required (Spatz and Strittmatter, 1973). Another example of autoactivation of a membrane-bound enzyme is the chitin synthetase zymogen of M. rouxii (Ruiz-Herrera and Bartnicki-Garcia, 1976). Activity of this enzyme increases in mixed membrane fractions due to the action of an endogenous protease. Although proteolytic release of this type was not demonstrated to be involved in release of Achlya cellulase, the decrease in total protein during incubation at RT indicates that this possibility is one of several that bear investigation.

UDPG Transferase

The high activity of UDPG transferase in Achlya walls is also a common observation in other fungi (Wang and Bartnicki-Garcia, 1966; McMurrough et al., 1971; Meyer et al., 1976; Fèvre and Dumas, 1977) and in higher plants (Shore and Maclachlan, 1975). In the closely related genus Saprolegnia (Fèvre and Dumas, 1977), about 45% of the activity is wall-bound, while in Achlya, the level is 42% (Table 10).

This observation is evidence in support of two assumptions regarding wall synthesis. First, it serves as an indication that these enzymes are indeed involved in cell wall metabolism. Second, it indicates that at least part of the cell's wall-synthesizing ability is indeed extracytoplasmic, and a considerable amount of wall assembly can

occur there. This requirement had been postulated on purely theoretical grounds, necessitated by the architectural complexity of the mature wall (Preston, 1974).

The fact that the products of isolated transferase preparations are often not rigorously identified (as indeed they remain in this investigation) lends itself to doubts concerning both the identity of the natural products and their exact roles in the cell. In addition, even well identified products produced *in vitro* may bear small resemblance to the *in vivo* products of the same enzyme(s), because any number of variable conditions can affect the nature of the products, and their levels in the microcompartments of the cell cannot be known.

Among these factors are the nature and concentration of the nucleotide-sugar donor (Ordin and Hall, 1968; Lamport, 1970; Tsai and Hassid, 1971, 1973), the presence of metal ions (Tsai and Hassid, 1973; Fèvre and Dumas, 1977), the presence of carbohydrate or alcohol activators (Thomas *et al.*, 1969; Spencer *et al.*, 1971; Southworth and Dickinson, 1975), and the presence of plant hormones (Van Der Woude *et al.*, 1972). The reason for much of this diversity almost surely lies in the poorly purified nature of the enzyme preparations. Undoubtedly, more than one type of enzyme is present which is capable of utilizing nucleotide sugar donors (Tsai and Hassid, 1971, 1973; Shore and Maclachlan, 1973).

A variable which may exert fundamental control over activity *in vivo* is the level of certain unidentified factors in the soluble phase of cell homogenates. In addition to proteolytic activators (Ruiz-Herrera and Bartnicki-Garcia, 1976), soluble inhibitors have been reported, and these may account for the low specific activity of many

soluble transferases. One such report is that by Fèvre and Dumas (1977) in S. monoica, where the specific activity of the combined soluble and particulate fractions was about 50% of that expected based on the activities in the separate fractions. In Achlya, however, no evidence of a soluble inhibitor is shown, even though the specific activity of soluble transferase is quite low. The specific activity of the combined soluble and particulate fractions is in fact about 9% higher than that expected on the basis of separate assays of the two phases (cf. Tables 10 and 11). Thus, no soluble inhibitor is indicated.

In the present investigation, the identity of transferase products was investigated only insofar as their solubility was concerned (Table 12). Most of the products are soluble either in hot water or hot alkali. Care must be taken not to place excessive emphasis upon the exact distribution of these products, however, because it cannot be assumed that each fraction represents a distinct class of molecules, nor that these solubilities correspond closely to those exhibited by extensive polymers of the types just synthesized. It is generally conceded that, in vitro, transferase enzymes are successful in transferring only a small number of sugars to the endogenous acceptor(s) (Preston, 1974). (Exceptions would be those chitin synthetases previously noted.) Thus, the solubilities of radioactive products are determined largely by the solubilities of the acceptors. It is to be hoped that the new linkages are formed using an acceptor of the corresponding type, but this has not been demonstrated.

A further complication arises, because the solubility exhibited by long and short polymers of the same material may vary. A β-1,4-oligoglucoside may be readily soluble in water, whereas insolubility

in most solvents is a hallmark of the longer cellulose I complexes (Whitaker, 1971). In some systems, the hydrolysis of soluble and insoluble fractions yields different products (Shore and Maclachlan, 1975). However, in others, these fractions may contain identical linkages, despite differences in solubility (Heiniger and Delmer, 1977).

Bearing these cautions in mind, it can be seen that the solubility-distribution of Achlya transferase products (Table 12) is comparable to that seen in S. monoica when 10 μM UDPG is used (Fèvre and Dumas, 1977). The main difference is in the lipid-soluble fraction, which constitutes a much greater proportion of the products in Saprolegnia. One reason may be that the ethanol-insoluble products served as the starting point for fractionation in the present research, whereas cold-water-insoluble products were fractionated in Saprolegnia. Probably, some lipid-soluble materials were lost in the ethanol washes.

Another source of discrepancy may lie in the 242 μM concentration of UDPG employed in the present study, compared to the 10 μM concentration used by Fèvre and Dumas (1977). As already mentioned, the concentration of the substrate is one of the many factors that can influence the nature of the products.

An attempt was made to characterize only the alkali-insoluble products in Saprolegnia; these yielded glucose and cellobiose upon hydrolysis, and the alkali-insoluble materials were identified as cellulose (Fèvre and Dumas, 1977). The close taxonomic affinity between the genera and the other similarities in the distribution and products of their respective transferases make it probable that the alkali-insoluble product of Achlya transferase also consists of a polymer with

β-1,4-glucosidic linkages. However, for the various reasons described, this cannot be certainly stated until the Achlya products are themselves analyzed.

THE ASSOCIATION OF WALL SYNTHESIS AND ENZYMES WITH HYPHAL GROWTH

Enzymes in the Culture Filtrate

Although it is probable that certain wall components are at least partially assembled inside the cell, the architecture of the wall requires that the final steps in assembly be extracellular (Preston, 1974). Enzymes involved in this assembly and others that may be present in secretory vesicles will thus be found outside the protoplast. These may eventually diffuse into the culture medium. To gain an indication of the kinds of enzymes secreted during growth, the filtrate from Achlya cultures was examined for enzyme activity. Enzyme activity in cell homogenates was also examined to serve as a basis for comparison with activities in the medium.

Methods

Two 48 hr old liquid cultures were harvested on miracloth, and both mycelium and filtrate were saved and pooled separately. After cooling the medium to 4 C, the filtrate was concentrated for 24 hr using a Millipore Immersible Molecular Separator (Millipore Corp.) equipped with a pellicon membrane with a 10,000 nominal molecular weight limit. When the volume had been reduced to 10 ml, samples were assayed for enzyme activities.

Mycelium was homogenized in 2 g FW lots by standard methods. Centrifugation (270 x g x 10 min) removed heavy material, and samples

of the homogenate were then assayed for enzyme activities. (Mycelial cellulase was assayed in the presence of 1% triton X-100.) Results are displayed in Table 13.

Results

Every enzyme assayed displays activity in both the mycelium and the culture filtrate. However, the only filtrate activities which are very high in comparison to their levels in the mycelium are those of cellulase and ATPase. The activities of acid phosphatase, cytochrome oxidase, and perhaps UDPG transferase are so low as to be insignificant compared to mycelial levels.

The presence of cellulase in Achlya culture media has been reported before (Bhargava, 1943; Thomas and Mullins, 1967, 1969). What is demonstrated in the current experiment is that cellulase is not alone in the medium; but, compared to its companion enzymes, cellulase activity is perhaps the highest of all. This observation warrants our continued investigation of the possible association between cellulase and growth in Achlya.

Comparisons of the Growing and Nongrowing Conditions

For the growth of Oomycetes, sufficient amounts of certain minerals, organic nitrogen, organic sulfur, and a suitable carbon source are required (Whiffen, 1945; Barksdale, 1962; Cantino, 1966). These requirements are met by the Defined Liquid Medium (DLM). Removal of nutrients will inhibit growth, and this is especially true of nitrogen depletion (Barksdale, 1962; Griffin et al., 1974). Complete removal of all nutrients, however, will induce sporangial development (Klebs,

TABLE 13. The activities of enzymes in the mycelium and culture filtrate of 48 hr old cultures of *A. ambisexualis*

Enzyme	Activity per g FW of mycelium*	Activity per flask (200 ml) of culture filtrate*	Filtrate activity / Mycelial activity
Acid phosphatase	377 ± 60	12 ± 0.2	0.0
Alkaline phosphatase	15 ± 3	6 ± 0.1	0.4
ATPase	30 ± 1	106 ± 4	3.6
Cellulase	68 ± 9	327 ± 3	4.8
Cytochrome oxidase	473 ± 5	2.4 ± 1.7	0.0
β-glucosidase	371 ± 4	286 ± 6	0.8
Glucose-6-phosphatase	38 ± 6	15 ± 1	0.4
IDPase	20 ± 3	14 ± 1	0.7
UDPG transferase	1149 ± 13	141 ± 46	0.1

*All activities are expressed as nmoles of substrate transformed per min, except the following: UDPG transferase, pmoles of glucose incorporated per min; cellulase, percent decrease in flow time of substrate per hr. Each value is the average of 3-4 measurements.

1899), which is accompanied within a short time by its own set of specialized physiological responses (Griffin and Breuker, 1969; Timberlake et al., 1973; O'Day and Horgen, 1974). These might be expected to interfere with the measurements of conditions in the non-growing state. Fortunately, sporangial formation can be avoided in a depleted medium by maintaining the level of glucose or other carbon source (P. A. Horgen, pers. comm.). This was confirmed by an experiment in which sporulation was attempted by the methods already described, but 0.5 mM $CaCl_2$ supplemented with 0.2% w/v glucose was used, instead of calcium chloride solution alone. No spores or sporangia were found; therefore, 0.2% glucose suppressed sporulation. Accordingly, the medium employed to eliminate growth in the following experiments was 0.2% w/v Glucose Medium (GM), adjusted to pH 6.9 with HCl. In addition to suppression of sporulation, this medium permits the study of radio-glucose uptake using the same concentration of glucose found in DLM.

Changes in Mycelial Fresh Weight During Incubation in DLM or GM

Methods

Mycelium from 48 hr old liquid cultures was harvested on miracloth and rinsed with 250 ml of deionized water. Excess water was removed by gentle pressure with a rubber spatula. The mycelium was divided into 2.0 g FW lots, which were resuspended in separate 250 ml Erlenmeyer flasks containing 50 ml of either DLM or GM. Flasks were incubated at 24 C with shaking and were harvested hourly on miracloth; excess water was removed as before. Mycelial mats were weighed, and the results are displayed in Table 14.

TABLE 14. Changes in fresh weight of 2 g FW lots of A. ambisexualis mycelium during incubation in Defined Liquid Medium (DLM) or 0.2% Glucose Medium (GM)

Time in hours	Fresh weight of mycelium in grams*	
	DLM	GM
0	2.0	2.0
1	2.11 ± 0.13	1.88 ± 0.25
2	2.73 ± 0.24	2.06 ± 0.09
3	3.14 ± 0.36	2.08 ± 0.08
4	3.25 ± 0.34	2.07 ± 0.15
5	4.07 ± 0.17	2.05 ± 0.23

*Each value is the average of 3-4 measurements.

Results

From hours 1 to 5, each original gram of mycelium increases its fresh weight at a rate of about 0.25 g/hr in DLM. In the same interval, each gram of mycelium in GM increases its fresh weight by only about 0.02 g/hr. Thus, nutrient depletion decreases the growth rate of Achlya mycelium by at least 90% within one hour, and the effect remains through 5 hr.

The Incorporation of Exogenous Glucose into Cell Walls of Growing and Nongrowing Mycelium

Methods

Two-gram lots of mycelium were obtained and resuspended in either DLM or GM, as before. These media were supplemented with 0.5 μCi of

uniformly labeled ^{14}C -glucose (New England Nuclear, 198 mCi/mmole). Mycelia were harvested hourly on miracloth, washed with deionized water, and transferred to 15 ml conical glass centrifuge tubes.

The following cell wall isolation is a modification of the method of Aronson et al. (1967). A 5.0 ml volume of methanolic KOH (5% w/v KOH; 80% v/v methanol) was added to each lot of mycelium in a conical centrifuge tube, and the mycelium was sonicated for 60 sec at a setting of 7 on the Heat Systems-Ultrasonics sonifier. The volume was increased to 10 ml with methanolic KOH, and the tube was heated at 70-80 C for 15 min. Mycelial fragments were centrifuged from the heated suspension at about 1,000 x g x 2 min. After discarding the supernatant, fragments were retreated as described. The second wall fragment pellet was resuspended in 10 ml of methanolic KOH, heated again for 15 min, and recentrifuged to yield a third pellet. This pellet was washed twice in 10 ml volumes of 80% methanol, followed by a 10 ml distilled water wash, and then the pellet was transferred to a scintillation vial in 3.0 ml of distilled water. After liquid scintillation counting, the amount of glucose incorporated into the wall fractions was calculated, and the results are displayed in Table 15.

Results

From hours 1 to 5, glucose is incorporated into walls at a rate of about 95 nmoles/min/g FW in DLM; while, in GM, the rate is about 12 nmoles/min/g FW. This represents about an 87% reduction in rate during this time. Thus, as expected, cell growth is accompanied by cell wall synthesis, and the rate of synthesis is comparable to the rate of growth.

TABLE 15. The incorporation of exogenous glucose into walls of 2.0 g FW lots of A. ambisexualis mycelium during incubation in Defined Liquid Medium (DLM) or 0.2% Glucose Medium (GM)

Time in hours	µmoles of glucose incorporated into mycelial walls*	
	DLM	GM
1	4.5 ± 1.4	3.4 ± 0.2
2	11.8 ± 1.7	6.6 ± 0.3
3	24.5 ± 6.5	6.8 ± 0.3
4	32.8 ± 1.3	7.1 ± 0.7
5	50.3 ± 7.6	9.2 ± 0.5

*Each value is the average of 3-4 measurements.

The Secretion of Cellulase during Mycelial Growth

As was seen in Table 13, cellulase is perhaps the most conspicuous enzyme found in Achlya culture media. To determine whether the rate of cellulase secretion is correlated with the growth rate, the appearance of cellulase in the medium was examined under controlled conditions of full growth and retarded growth.

Methods

Two-gram lots of mycelium were obtained and resuspended in DLM or GM, as in the preceding experiments. After hourly periods of growth, mycelia were harvested and discarded; the liquid media were treated to extract cellulase by the method of Thomas and Mullins (1967, 1969). Media were made 5% in NaCl, followed by addition of 5 volumes of 95%

ethanol. The cloudy liquids were centrifuged at 16,000 x g x 20 min, and the supernatants were discarded. Sediments were drained of ethanol and resuspended in 2.0 ml of distilled water. These resuspended pellets were centrifuged at 27,000 x g x 15 min, and supernatants were saved for assay of cellulase activity. Results are displayed in Table 16.

TABLE 16. The recovery of cellulase from media after incubation of 2.0 g FW lots of A. ambisexualis mycelium in 50.0 ml volumes of Defined Liquid Medium (DLM) or 0.2% Glucose Medium (GM)

Time in hours	Units of cellulase per 50 ml of medium*	
	DLM	GM
1	11.7 ± 4.0	3.7 ± 1.0
2	21.9 ± 5.6	4.8 ± 1.7
3	40.8 ± 10.0	3.9 ± 0.9
4	49.3 ± 7.0	1.6 ± 0.1
5	57.1 ± 15.4	3.7 ± 1.6

*Each value is the average of 3-4 measurements.

Results

Cellulase is secreted by growing mycelia at a rate of about 5.7 units/hr/g FW from 1 to 5 hours, whereas net secretion of cellulase by nongrowing mycelia cannot be detected during this same period. There is, therefore, a very close correlation between the secretion of cellulase into the medium and growth of the mycelium. It cannot, of course, be stated at this time which event, if any, is a requirement for the other.

The Specific Activities of Mycelial Enzymes during Incubation of Mycelia in DLM or GM

It would be interesting to know whether the lack of cellulase secretion observed in nongrowing mycelia is accompanied by a lack of intracellular enzyme as well. To that end, the specific activities of cellulase in growing and nongrowing mycelia were determined. In addition, the specific activities of other enzymes and the amount of carbohydrate/mg protein in these mycelia were determined.

Methods

Two-gram lots of mycelium were resuspended as in the preceding experiments in either DLM or GM and incubated in these media for 3 hours. (In preceding experiments, the patterns of growth, wall synthesis, and cellulase secretion were seen to be already established by T = 3 hr.) Mycelia were homogenized as described previously, and both soluble and particulate fractions were assayed for carbohydrate content, protein content, and enzyme activities. (Carbohydrate content in the soluble phase is not reported, because of the interference of the sucrose osmoticum. Particulate cellulase was assayed in the presence of 1% triton X-100.) Specific activities and carbohydrate per mg protein were calculated; and, where these values differed between growing and nongrowing mycelia, the degree of confidence in that difference was determined. Results are displayed in Table 17.

Results

For protein content, carbohydrate content, and the specific activities of ATPase, IDPase, and UDPG transferase, there is no statistically significant difference between overall levels in the growing

TABLE 17. Protein content, carbohydrate content, and the specific activities of mycelial enzymes in the particulate and soluble phases of A. ambisexualis mycelial homogenates, after incubation of 2 g FW lots of mycelium for 3 hr in either Defined Liquid Medium (DLM) or 0.2% Glucose Medium (GM)

Compound or activity assayed	Particulate activity			Soluble activity		Total activity		
	DLM	GM		DLM	GM	DLM	GM	
Protein[1]	0.94 ±0.06	1.03 ±0.07		1.06 ±0.09	0.90 ±0.09	1.99 ±0.14	1.93 ±0.14	
Cellulase[2]	66.6 ±3.2	32.9 ±4.9	.01*	19.4 ±2.1	18.9 ±1.9	40.9 ±2.2	23.8 ±1.3	.02*
Carbohydrate[3]	221 ±5	228 ±4		N.D.	N.D.	N.D.	N.D.	
Acid phosphatase	236 ±3	419 ±24		50 ±14	107 ±24	137 ±10	273 ±23	.01*
β-glucosidase	110 ±2	134 ±3		153 ±6	224 ±2	132 ±2	174 ±1	.01*
Alkaline phosphatase	0.77 ±0.06	1.07 ±0.17		18.0 ±0.1	9.2 ±0.1	9.7 ±0.7	4.4 ±0.2	.02*
Cytochrome oxidase	129 ±1	166 ±5		0.0	0.0	65 ±1	83 ±3	.01*

TABLE 17 - continued.

Compound or activity assayed	Particulate activity			Soluble activity		Total activity		
	DLM	GM		DLM	GM	DLM	GM	
Glucose-6-phosphatase	20.4 ± 2.2	52.4 ± 1.5		3.6 ± 0.7	16.0 ± 0.7	11.7 ± 1.5	36.4 ± 1.2	.01*
ATPase	16.2 ± 1.7	19.7 ± 3.3		7.3 ± 1.0	10.1 ± 1.5	11.4 ± 1.3	15.0 ± 0.9	
IDPase	15.8 ± 2.0	12.9 ± 2.5		8.7 ± 0.3	13.6 ± 1.6	12.1 ± 1.2	13.1 ± 0.6	
UDPG transferase[4]	1099 ±22	920 ±23	.05*	130 ±11	121 ± 5	566 ±16	520 ± 9	

*Confidence Level from Student's "t" test

N.D. - Not Determined

[1]mg per g FW

[2]units per mg protein

[3]µg per mg protein

[4]pmole per min per mg protein

Except as noted, all activity is expressed as nmole per min per mg protein.

Each value is the average of 3-4 measurements.

and nongrowing states. For UDPG transferase, there is a slight, but significant, increase in particulate specific activity in growing mycelia. Those enzymes showing significantly higher overall specific activities in the nongrowing state are acid phosphatase, β-glucosidase, cytochrome oxidase, and glucose-6-phosphatase.

Only two of the tested enzymes exhibit clear-cut increases in specific activity in the growing state. These are cellulase and alkaline phosphatase. In the growing state, over 95% of alkaline phosphatase activity is in the soluble fraction, which is in agreement with the already described observation that no subcellular structures react positively to the cytochemical test for this enzyme. The overall increase in cellulase specific activity is based on differences in specific activities in the particulate phase, where specific activity of cellulase is doubled in growing mycelia.

Discussion

Every mycelial enzyme investigated can also be found in the culture filtrate. Investigations of nutrition in fungi (Bhargava, 1943; Reese, 1959), of reproductive development (Thomas, 1966; O'Day and Horgen, 1974), and of fungal cell wall metabolism (Cortat et al., 1972; Fèvre, 1972; Mullins, 1973; Meyer et al., 1976) have reported the appearance of various specific enzymes in culture media, but most of these investigations have been concerned only with a single enzyme. One investigation of wall metabolism that studied a variety of released enzymes is that by Meyer et al. (1976), who studied growth in Phytophthora. Arbitrarily measured levels of activity were reported for β-1,3-glucanase, β-glucosidase, acid phosphatase, and alkaline phosphatase. Levels of

UDPG transferase, α-mannosidase, and β-galactosidase were reported as insignificant.

Unless very standardized assays are employed, the actual units of activity reported by Meyer et al. (1976) or in any other investigation mean very little. For instance, when it is reported that Phytophthora culture filtrates contain "2.8 arbitrary units per mg protein" of acid phosphatase activity, while UDPG transferase activity is "insignificant," one is forced to rely on the investigators' experience that the activities in question are "high" or "low."

In the present study, enzyme activities in Achlya filtrates are compared to activities found in an arbitrarily chosen amount of mycelium (1 g FW). By this method, it can be seen that the activities of ATPase and cellulase in each flask of filtrate are from 3 to 5 times greater than their activities in each gram of mycelium. Ratios of other enzyme activities are less than unity. If enzyme activities alone had been reported, it would have been impossible to state whether cellulase activity is significant in comparison to the other enzymes, because cellulase is the only enzyme measured in arbitrary units. The activity of β-glucosidase may be the greatest of all enzymes measured, in terms of absolute enzyme units. But, when compared to its mycelial levels, it is present in "average" amounts in the filtrate. Thus, while the high absolute activity of β-glucosidase must be acknowledged, the disproportionately high levels of cellulase and ATPase are perhaps more significant.

Care must be taken in interpreting these results, because the stability of each enzyme plays a role in determining its activity in the filtrate. During growth, the medium was incubated at room

temperature for two days, followed by about 24 hr at 4 C during concentration. Even at 4 C, the stabilities of UDPG transferase and cytochrome oxidase were observed to be poor, and this undoubtedly contributed to their low activities in the filtrate. Furthermore, enzymes in the filtrate were assayed in the presence of metal ions from the medium. This might be particularly important in the case of ATPase; certain forms of this enzyme are sensitive to levels of Mg^{++}, Na^{+}, and/or K^{+} ions (Hodges and Leonard, 1974), all of which are present in the filtrate. This could conceivably account for the very high level of ATPase activity in the filtrate.

The secretion of cellulase is correlated both with cell growth and with wall synthesis, and all three processes cease under nutrient stress (Tables 14, 15, 16). Several other cases of cell growth, such as hyphal branching (Mullins, 1973) and budding of yeast (Cortat et al., 1972), are also associated with enhanced levels of glucanase secretion. Another correlation between growth and cellulase lies in the intracellular levels of this enzyme (Table 17). The difference is seen only in the particulate phase (and this is another indication that the particulate and soluble pools are independent). The abolition of secretion after incubation in GM is virtually complete by the second hour; and yet the specific activity of mycelial cellulase is not abolished, though its overall level is reduced by about 42% (Table 17). This indicates that secretion ceases, not for lack of mycelial cellulase, but because of some alteration in the mechanism for secretion or the availability of energy for secretion. A comparable situation may exist in yeast, where exponential growth is accompanied by a doubling of the levels of cellular β-1-3-glucanase over the level seen during stationary growth.

Secretion of the same enzyme undergoes about a twelve-fold increase during exponential growth (Cortat et al., 1972).

Cellulase and alkaline phosphatase are the only mycelial enzymes whose overall activities were significantly higher in the growing state. While particulate UDPG transferase is also significantly higher in growing mycelia, it is an increase of only about 19%, compared to 72% and 120% increases in overall cellulase and alkaline phosphatase activities, respectively. The imbalance between the levels of wall synthesizing and wall degrading enzymes is consistent with those models of growth in which regulation is achieved by a balance between synthesis and lysis (Park and Robinson, 1966; Bartnicki-Garcia, 1973).

The role, if any, of alkaline phosphatase in this process is not apparent. The possibility exists that some regulatory role is played by this apparently soluble enzyme, and this should be investigated.

Certain enzymes displayed significantly higher activity in the nongrowing state. For two, acid phosphatase and β-glucosidase, a reason may lie in their common association with general degradative metabolism (Wattiaux, 1969). While detailed studies of the physiology of Achlya under nutrient stress have not been made, these enzymes might be expected to contribute to the mobilization of internal nutrient stores. Glucose-6-phosphatase in mammalian systems functions to remove phosphoglucose from the glycolytic pools of phosphorylated sugars (White et al., 1959), though no reason is apparent for an increase in its specific activity in nongrowing cells of Achlya. The increase in cytochrome oxidase activity is surprising, since one might reasonably expect oxidative metabolism to be depressed under nutrient stress.

Finally, a comparison can be made between the rate of glucose incorporation into products by isolated UDPG transferase and the rate of incorporation of glucose into walls of intact growing cells. The rate of incorporation due to isolated transferase is about 3.6×10^{-9} moles·min^{-1} per g FW (calculated from Table 10). The rate of incorporation into growing walls can be calculated as 0.9×10^{-7} moles·min^{-1} per g FW (Table 15). Only about 4% of the rate of incorporation into growing walls can be accounted for by isolated wall-bound and cytoplasmic synthetase activity. This is in agreement with Maclachlan's (1976) observation that, at best, usually only about 5% of the rate of wall incorporation can be accounted for in this way.

ISOLATION OF CELLULASE-CONTAINING MEMBRANES

In the preceding experiments, the association of mycelial cellulase with cellular membranes was demonstrated, though the identity and nature of these membranes is not known. In hopes of determining how many types of cellulase-rich particles exist, their characteristics, and their identities, a number of experiments were conducted. In particular, the isopycnic density of cellulase-rich particles was examined, as was their association with other enzyme activities.

The Distribution of 280 nm-absorbing Materials and Cellulase-containing Particles in Isopycnic Sucrose Gradients

The Distribution of 280 nm-absorbing Materials in an Isopycnic Sucrose Gradient

Each cellular particle exhibits its own buoyant density; and, though the exact density of individual particles in a population may differ slightly, homogeneous populations usually exhibit a mean density that is characteristic of the particle class. By centrifuging a mixture of different particles in a solution exhibiting a gradient of densities, classes of particles can be segregated to a degree dependent on differences in their buoyant densities. This occurs because each particle will migrate in the gradient until it encounters a buoyant density equal to its own. At that point, both gravitational and buoyant forces counter-balance; the particle is said to have reached its isopycnic point, and migration ceases (Tolbert, 1974). The following experiments

are designed to reveal the isopycnic point(s) of cellulase-containing particles.

Methods

A mycelial homogenate was obtained by grinding the contents of 48 hour old liquid cultures with a mortar and pestle, as described previously. However, since various steps in purification are accompanied by considerable loss of material, it was necessary to begin with larger amounts of mycelium than those used in the preceding experiments. And, for certain experiments, it was felt desirable that homogenates occupy a relatively small volume. Therefore, in each homogenization, mycelia from three separate cultures were ground together in the same mortar with 5.0 ml of homogenizing solution. This was of the same composition at that previously used (i.e., 0.03 M tris·HCl buffer, pH 7.6; 30% sucrose) but with 15 mM DTT and 0.3% BSA. This last ingredient was added as a precautionary measure because of its value during homogenization in protecting against certain degradative enzymes and free fatty acids (Galliard, 1974). DTT was included because many synthetase enzymes have been found to require its presence during isolation (Preston, 1974). As in previously described homogenizations, a second grinding employed a small volume (usually about 5 ml) of one-third-strength homogenizing solution.

After removal of sand and cellular debris by centrifuging at 270 x g x 10 min, the homogenate was adjusted so that its weight per centrifugation tube was 12.0 g, and its sucrose concentration was 12-13%, as determined by a Bausch and Lomb refractometer. The homogenate was then layered atop a linear sucrose gradient.

Gradients were constructed with a simple gravity flow gradient maker and contained 5 mM DTT and 0.01 M tris·HCl buffer, pH 7.6. Sucrose concentration extended from 20% (ρ=1.08 g/cm^3) to 55% (ρ=1.26 g/cm^3) over a 1 ml cushion of 65% sucrose. The total volume of gradient and cushion was 25 ml in a 38.5 ml cellulose nitrate tube. This left room for the entire 12.0 g of homogenate to be layered atop the gradient.

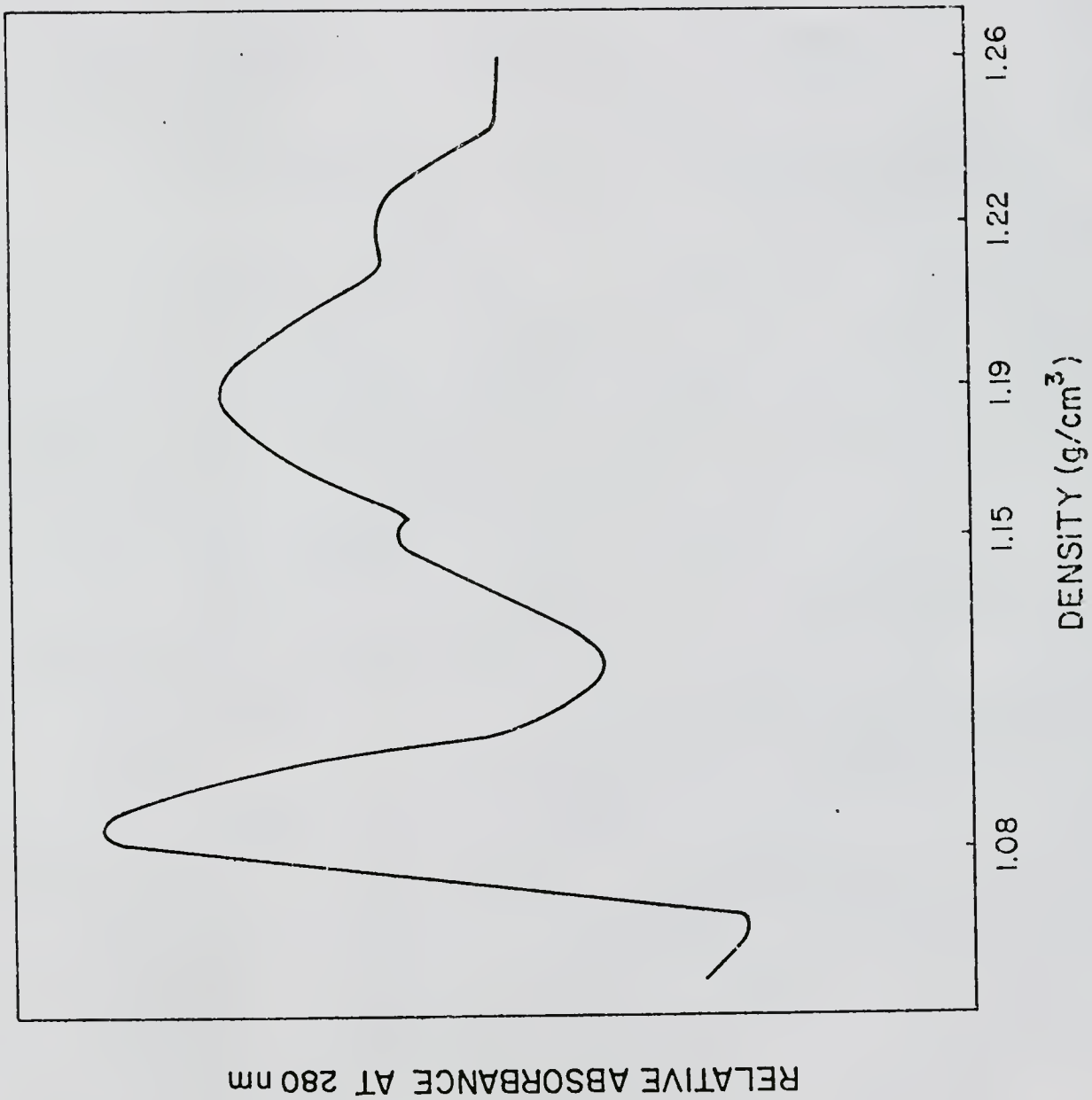
Gradients were centrifuged in a Spinco SW 27 rotor at 112,000 x g x 3 hr. (Preliminary experiments indicated that the isopycnic point had been reached by this time.) Fractions were collected drop-wise by puncturing the bottom of the centrifuge tube, and the absorbance at 280 nm was monitored by an in-line LKB Uvicord II 8300 UV analyzer. Sucrose concentrations of fractions were determined refractometrically. All steps were performed at 4 C.

Results

The distribution of 280 nm-absorbing materials in a sample gradient is shown in Figure 23. Four major peaks of absorbance are revealed; however, the peak at a density of 1.08 g/cm^3 seems to be composed of materials which, for some reason, were retarded at the interface between the homogenate and the gradient. This was demonstrated by constructing an identical gradient in which the homogenate was evenly distributed before centrifugation, rather than being layered on top. In this case, centrifugation produced the three peaks at densities from 1.15 to 1.22 g/cm^3, but the 1.08 g/cm^3 peak was missing.

The three peaks of interest, then, are those crowded closely together, with mean isopycnic densities of 1.15 g/cm^3, 1.19 g/cm^3, and

Fig. 23. The distribution of 280 nm-absorbing materials after centrifugation to equilibrium of the 270 x g x 10 min supernatant from an A. ambisexualis homogenate in a linear sucrose gradient


RELATIVE ABSORBANCE AT 280 nm
1.08
1.15
1.19
1.22
1.26
DENSITY (g/cm³)

1.22 g/cm^3. The relative heights of these peaks varied from experiment to experiment, though the 1.19 g/cm^3 peak was usually the most prominent. On many occasions, this peak completely overshadowed the others, rendering them unresolvable on the UV scan. On a few occasions, scans revealed that the 1.19 g/cm^3 peak may in fact be composed of two subpeaks equilibrating at densities of about 1.18 and 1.20 g/cm^3, respectively. That these two peaks are not commonly resolved may perhaps result from one peak's predominance over the other.

The Distribution of Cellulase-containing Particles in Isopycnic Sucrose Gradients

Methods

Using the same methods as those of the preceding experiment, particles from homogenates were centrifuged to isopycnic equilibrium, and gradients were fractionated as described. After refractometric determination of sucrose concentration, fractions were diluted with about two volumes of gradient solution made 20% in sucrose, and particulate material was pelleted at 79,000 x g x 60 min in the Spinco 65 rotor. Sediments were resuspended in 2.0 ml of distilled water, and aliquots were removed for assays. Before carbohydrate assays, aliquots were recentrifuged, as described above, to remove residual sucrose. Aliquots to be assayed for cellulase were made 1% in triton X-100. Results are shown in Figures 24A and 24B.

Results

As Figures 24A and 24B reveal, there is considerable overlap between the distributions of various enzymes, indicating either that particles of any one class exhibit a wide range of densities or that

Fig. 24A. The distribution of cytochrome oxidase, IDPase, β-glucosidase, and UDPG transferase after centrifugation to equilibrium of the 270 x g x 10 min supernatant from an A. ambisexualis homogenate in a linear sucrose gradient

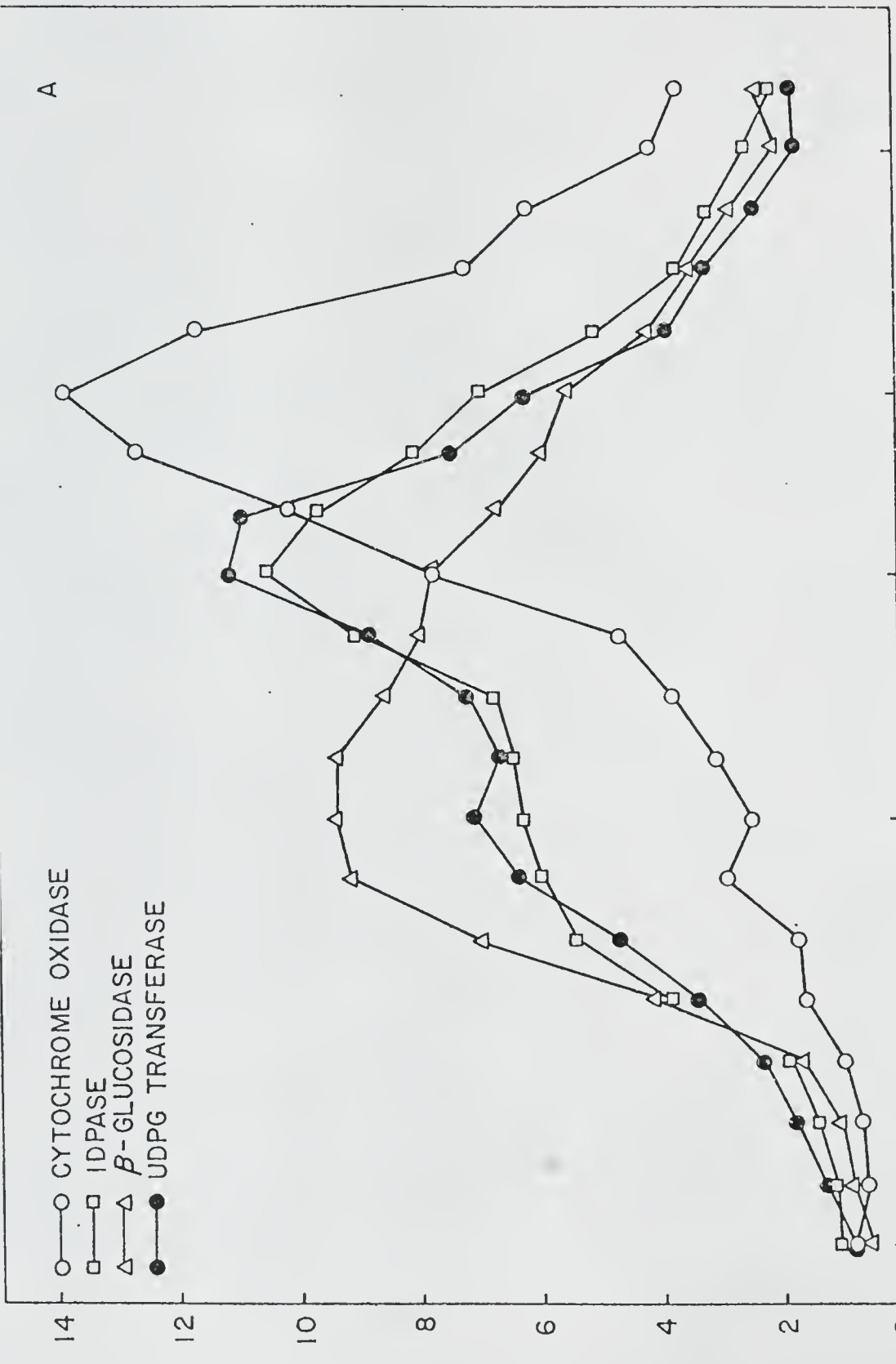
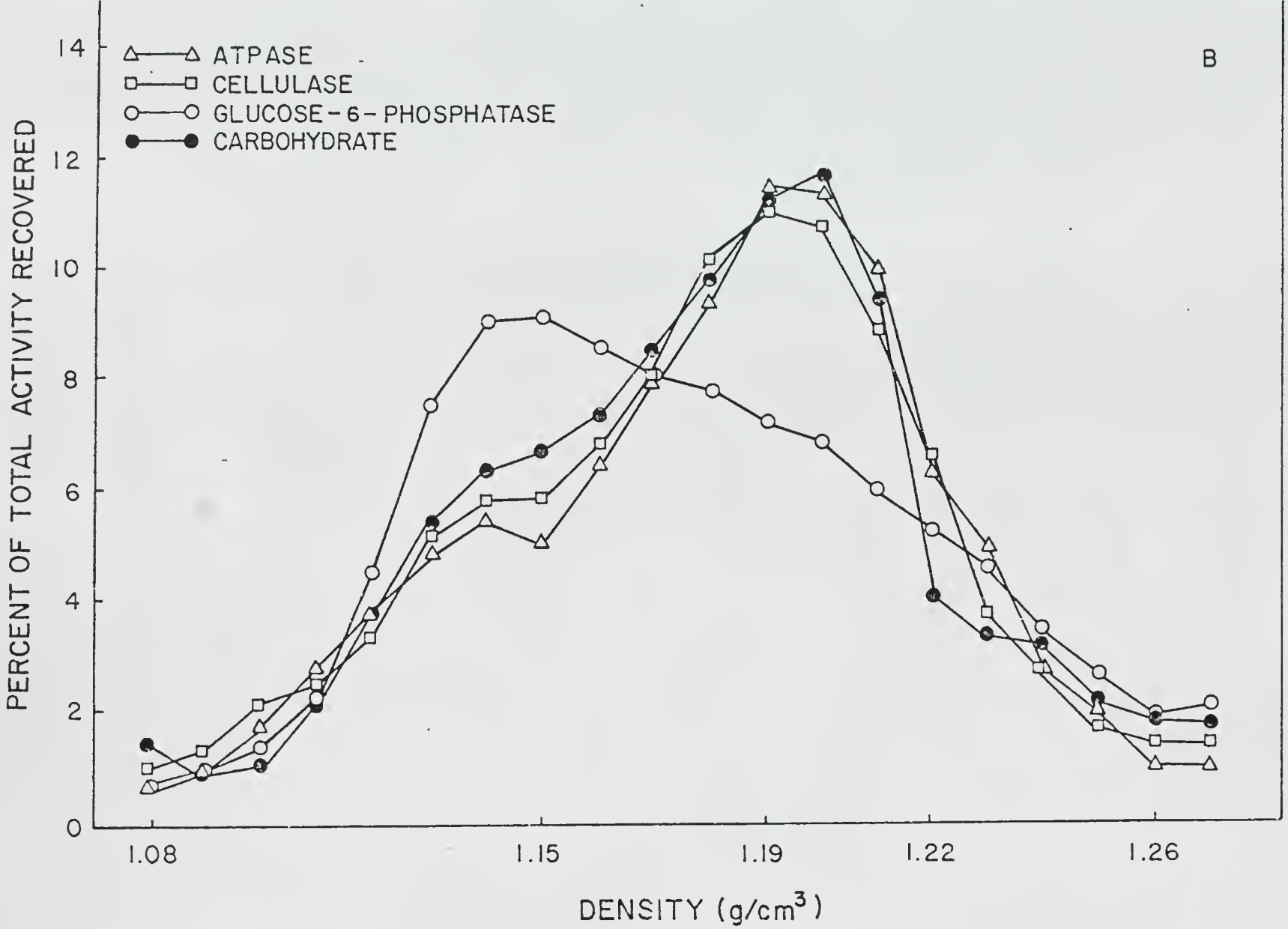

A
CYTOCHROME OXIDASE
IDPASE
β-GLUCOSIDASE
UDPG TRANSFERASE
PERCENT OF TOTAL ACTIVITY RECOVERED
0
2
4
6
8
10
12
14
1.08
1.15
1.19
1.22
1.26
DENSITY (g/cm³)

Fig. 24B. The distribution of ATPase, cellulase, glucose-6-phosphatase, and carbohydrate after centrifugation to equilibrium of the 270 x g x 10 min supernatant from an A. ambisexualis homogenate in a linear sucrose gradient


ATPASE
CELLULASE
GLUCOSE-6-PHOSPHATASE
CARBOHYDRATE
B
PERCENT OF TOTAL ACTIVITY RECOVERED
0
2
4
6
8
10
12
14
1.08
1.15
1.19
1.22
1.26
DENSITY (g/cm3)

many of the enzymes in question may be borne by particles of more than one type. Each case seems likely; the latter seems certainly to be true in view of the patterns displayed. Note that UDPG transferase activity, for example, is associated with both a major and a minor location, and that activities of glucose-6-phosphatase and β-glucosidase show strong "shoulders" in the region of particles equilibrating at 1.19 g/cm^3. Nevertheless, despite the heterogeneity of particle densities and the complication of multiple carriers of enzymes, the enzyme distributions do exhibit distinct maxima at densities corresponding to those of the previously described absorption peaks (Fig. 23). The cytochrome oxidase peak equilibrates at a density of around 1.22 g/cm^3, indicating that this region contains mitochondria or mitochondrial fragments. Similarly, the material at or about a density of 1.15 g/cm^3 is characterized by a high content of β-glucosidase and glucose-6-phosphatase. Finally, the central peak around 1.19 g/cm^3 contains most of the particulate cellulase, UDPG transferase, carbohydrate, ATPase, and IDPase activity. There are also strong shoulders of these activities associated with the 1.15 g/cm^3 material. Acid phosphatase activity (not shown) was irregularly distributed in the region of 1.15 to 1.19 g/cm^3, with no distinct peak.

In a separate experiment, gradient fractions were assayed for IDPase activity both before and after a 3-day incubation at 4 C. The activity declined in all fractions by about 5%. Cold-latent IDPase activity has been reported to be a biochemical marker for dictyosomes in some systems (Ray et al., 1969).

The carbohydrate in the 1.19 g/cm^3 peak was not biochemically identified, but its absorption spectrum was determined after reaction

with the anthrone reagent. A single absorbance peak was found at 625 nm, which is characteristic of glucose, mannose, and galactose. If the main carbohydrate had been ribose, absorbance peaks at 510 and 638 nm would have been expected (Herbert et al., 1971). Thus, while the identity of the carbohydrate is not known, ribose from membrane-bound ribosomes does not contribute significantly.

Finally, the ATPase with peak activity around 1.19 g/cm^3 was assayed with and without Na^+ and K^+ ions present to check for stimulation (Marriott, 1975). No stimulation was observed.

Since there is such a great degree of overlap between enzyme activities, and because materials equilibrating in the region of 1.19 g/cm^3 exhibit such a multiplicity of activities, further experiments were conducted in hopes of effecting better particle separations.

Enrichment of Cellulase-containing Particles by Sequential Differential, Velocity, and Isopycnic Centrifugation

The Separation of Enzyme Activities by Differential Centrifugation

This method employs differences in the mass and volume of particles to achieve separation. Clean separations are not common, however, because large differences in sedimentation rates are not usually encountered, but enrichments of fractions and information on the relative size distribution of materials in the homogenate can reasonably be anticipated. In hopes of achieving a "cleaner" preparation of cellulase-containing particles, the distribution of cellulase in differential fractions of homogenates was investigated. The distribution of glucose-6-phosphatase and cytochrome oxidase was also determined, because of their associations with particles of densities 1.19 g/cm^3 and 1.22 g/cm^3, respectively.

Methods

Mycelium was homogenized by the same method used to prepare homogenates for isopycnic centrifugation; however, BSA was omitted in order to minimize its interference with the determination of specific activities. After removal of 500 x g x 10 min material, homogenates were adjusted to 12.0 g and 12-13% sucrose. These were then centrifuged progressively at 1,000 x g x 10 min; 5,000 x g x 10 min; 10,000 x g x 10 min; 15,000 x g x 10 min; 25,000 x g x 10 min; and 37,000 x g x 90 min, which yielded six sediment fractions and a 37,000 x g supernatant. Particulate fractions were resuspended in distilled water, and the protein content and activities of enzymes in each fraction were determined. Cellulase activity was measured in the presence of 1% triton X-100. Results are displayed in Table 18.

Results

The highest specific activity of cellulase is found in the fractions sedimenting at 10,000 x g, 15,000 x g, and 25,000 x g. Although the greatest amount of cellulase activity is found in other fractions, those fractions would seem to be more heavily contaminated with other materials, resulting in lower specific activities.

Contamination with the mitochondrial marker, cytochrome oxidase, is significantly decreased in the 10 K, 15 K, and 25 K fractions, but activity of glucose-6-phosphatase was not reduced in these fractions. This is not surprising, since some glucose-6-phosphatase activity was associated with the 1.19 g/cm^3 (cellulase) peak on isopycnic gradients.

TABLE 18. Distribution of cellulase, cytochrome oxidase, and glucose-6-phosphatase in differential centrifugation fractions of A. ambisexualis mycelial homogenates

Fraction	mg protein†	Cellulase		Cytochrome oxidase		Glucose-6-phosphatase	
		units†	sp. act.*	$\frac{\text{nmole}}{\text{min}}$ †	sp. act.*	$\frac{\text{nmole}}{\text{min}}$ †	sp. act.*
1K x g	3.01	9.0	22.9	1773	588	58.0	39
5K x g	2.72	133.4	49.1	649	339	110.4	81
10K x g	1.86	146.2	78.8	162	87	101.6	110
15K x g	0.95	96.7	102.3	97	103	60.4	128
25K x g	0.86	71.7	83.9	86	100	47.7	111
37K x g	4.71	171.3	36.4	162	35	108.0	46
"soluble"	14.76	157.9	10.7	0	0	111.0	15

†Each value is the average of two measurements.

*Specific activities are expressed as units per mg protein.

The Behavior of Cellulase-containing Particles in a Velocity Gradient

A third method of particle purification is the velocity gradient, which, like differential centrifugation, separates particles on the basis of differences in their sedimentation rates. A distinction, however, is that the particles to be separated are not evenly distributed in a solution, but are layered atop a shallow density gradient. The gradient exhibits a range of densities that does not encompass the isopycnic densities of the particles under investigation.

In a centrifugal field, the particles would be capable of migrating completely through the gradient, but each will do so at a rate determined by its individual sedimentation velocity. Thus, lighter particles will tend to lag behind the heavier ones. If centrifugation is stopped before the particles of interest have passed completely through the gradient, they can be recovered from gradient fractions and will have been freed of distinctly heavier or lighter contamination to some degree. The function of the gradient is to stabilize the leading and trailing edges of particle bands as they develop and to minimize mixing during acceleration (Reid and Williamson, 1974).

Methods

Cellulase-containing particles were obtained by differential centrifugation of homogenates, as described in the previous section. The 10,000 x g, 15,000 x g, and 25,000 x g differential fractions were combined in 1.0 ml of gradient solution made 12 to 13% in sucrose, and this volume was layered atop a sucrose gradient with limits of 15% and 35% sucrose overlaying a 65% sucrose cushion. The total volume of the gradient and cushion was 15 ml in a 17 ml cellulose nitrate centrifuge

tube. Centrifugation was at 60,000 x g x 20 min in a Spinco SW 27.1 rotor, and fractions were collected dropwise from the bottom of the tube. These were sedimented at 79,000 x g x 60 min, as described earlier for isopycnic fractions, and assayed for cellulase in the presence of 1.0% triton X-100. Results are shown in Figure 25.

Results

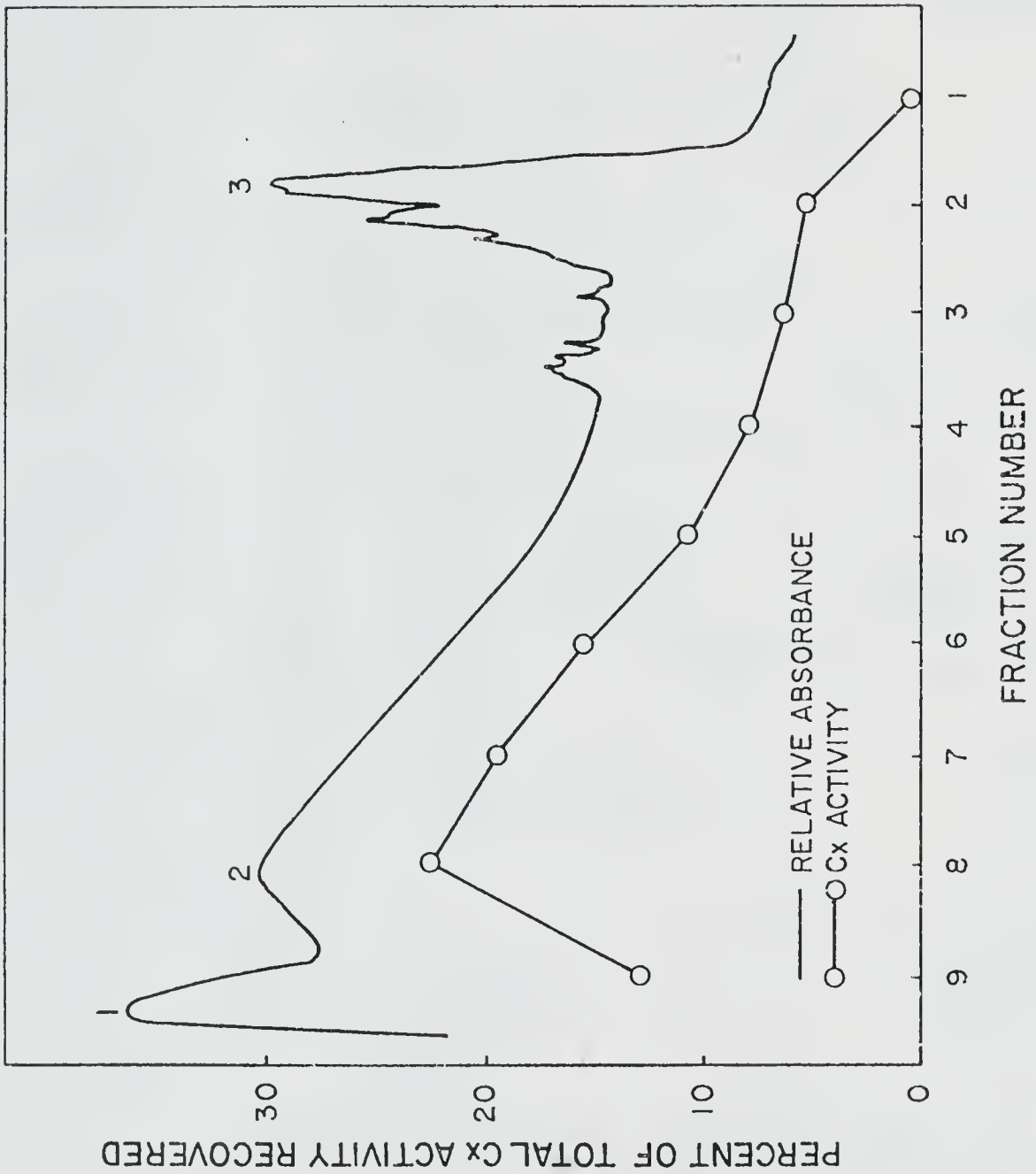
Most of the cellulase from the differential fraction is located in the center of the gradient and corresponds to the major peak of 280 nm-absorbing material (peak #2). The absorbance peaks at the bottom and top of the gradient represent minor contaminants of distinctly greater and lesser sedimentation rate, respectively. The cellulase-rich region reveals only a single average sedimentation rate with no evidence of subpopulations.

The density of these particles was investigated by layering fractions from the cellulase-rich peak #2 of a velocity gradient atop an isopycnic gradient constructed as described earlier. The particles (not shown) formed a single band with an average density of about 1.19 g/cm^3. Thus, it can be said that cellulase-rich particles sedimenting from homogenates between 5,000 x g x 10 min and 25,000 x g x 10 min are apparently homogeneous with regard to their behavior in velocity and isopycnic gradients.

The Enrichment of Cellulase-containing Particles by Differential, Velocity, and Isopycnic Centrifugation

Evidence from the preceding experiments indicates that the cellulase-containing particles observed in the major peak from isopycnic gradients can be purified to some degree by prior fractionations employing

Fig. 25. The distribution of cellulase and 280 nm-absorbing materials after velocity centrifugation of the "25 K x g" differential centrifugation fraction of an A. ambisexualis homogenate in a 15-35% linear sucrose gradient over a 65% sucrose cushion.


PERCENT OF TOTAL Cx ACTIVITY RECOVERED
0
10
20
30
1
2
3
RELATIVE ABSORBANCE
Cx ACTIVITY
0
1
2
3
4
5
6
7
8
9
FRACTION NUMBER

sedimentation rates. Furthermore, particles from this enrichment retain their isopycnic behavior and equilibrate at about 1.19 g/cm^3 in isopycnic gradients. To demonstrate the degree to which cellulase-containing membranes can be enriched by these methods, the process described in the previous experiment was repeated with a much larger amount of material. The specific activities of selected enzymes and the carbohydrate content were monitored at each stage in the process.

Methods

The mycelium from twelve culture flasks was homogenized in four separate groups, using the standard method. Cellulase-rich differential fractions were centrifuged as previously described on three separate velocity gradients, and selected fractions from the peak #2 region were pooled and diluted to a sucrose concentration of 19%. This entire volume was layered atop a 15 ml sucrose gradient (20-55%) in a 38.5 ml centrifuge tube and centrifuged isopycnically. Material was reserved for assay from the homogenate, the "5 K - 25 K" differential fraction, the velocity peak #2, and the pooled isopycnic fractions. Particles in these fractions were sedimented at 79,000 x g x 60 min and resuspended in water for assay. (Note that values for the homogenate fraction only represent the particulate component.) Results are displayed in Table 19. A portion of the material from the isopycnic band was kept aside and pelleted separately. This was prepared for electron microscopy, and observations are shown in Figures 26-31.

TABLE 19. Carbohydrate content and the specific activities of selected enzymes at each stage in the enrichment of cellulase-rich particles from A. ambisexualis mycelial homogenates.

Activity or material assayed	Homogenate	"5K - 25K" differential fraction	Peak #2 of velocity gradient	1.19 g/cm^3 isopycnic peak
Carbohydrate[1]	115	301	381	426
ATPase	34.2	74.3	91.0	105.5
Cellulase[2]	32.6	104.4	131.9	153.7
Cytochrome c oxidase	88.3	51.5	31.9	30.6
Glucose-6-phosphatase	43.7	109.2	113.5	116.1
IDPase	14.8	22.2	27.7	34.3
UDPG transferase	0.7	2.2	3.1	3.4

[1] μg carbohydrate·mg protein^{-1}

[2] units·mg protein^{-1}

Except as noted, all activity is expressed as nmoles of substrate transformed per min per mg protein. Each value is the average of two measurements.

Results

As can be seen from Table 19, velocity and isopycnic centrifugations serve to enrich cellulase-containing particles to a specific activity some 47% above that of the differential fraction. Compared to the specific activity of cellulase in membranes of the homogenate, enrichment is about 5-fold. Clearly, the single most effective step in this series is the differential centrifugation, which yields about a 3-fold increase in specific activity.

For the enzymes cytochrome oxidase and glucose-6-phosphatase, behavior is largely as expected. Cytochrome oxidase, which was shown to be largely removable by differential centrifugation (Table 18) and which equilibrates at 1.22 g/cm^3 (Fig. 24A), decreases in specific activity. Glucose-6-phosphatase, which was associated with materials at both 1.15 and 1.19 g/cm^3 in isopycnic centrifugations of homogenates (Fig. 24B), increases in specific activity with the differential centrifugation but exhibits only about a 6% increase over the next two steps. This is consistent with behavior which might be expected of an enzyme that is found in two classes of particles, one of which is being selectively enriched.

Carbohydrate and the remaining enzymes, all of which were originally associated mainly with the 1.19 g/cm^3 peak of isopycnic gradients of whole homogenates (Figs. 24A and 24B), increase in specific activity with each step in the enrichment. After the differential centrifugation, which gives varying degrees of enrichment, subsequent manipulations give about a 42% to 56% enrichment of specific activities for UDPG transferase, carbohydrate, IDPase, and ATPase over the specific activities in the differential fraction.

Electron microscopy of the enriched fraction of cellulase-containing membranes reveals that both dictyosome cisternae and unidentified smooth membranes are present (Figs. 26, 27). In addition, many membranes bear electron-dense particles which are about 20 nm in diameter (Fig. 28). Poststaining of sections with aqueous 0.5% uranyl acetate (not shown) selectively stains these particles, and this is evidence that they are ribosomes (Hayat, 1970). Most likely, these ribosomes are nonspecifically associated with membranes, because membrane-bound ribosomes would not be expected to retain their association with rough ER in a homogenizing solution that lacks Mg^{++} ions (Shore and Maclachlan, 1975). To what extent this nonspecific attachment may have altered the sedimentation properties of the membranes is not known, and further investigations should endeavor to eliminate this effect.

Staining of thin sections of the pellet by the PASM method shows that the major PASM-positive particles are the dictyosome cisternae and some (but not all) of the smooth membrane vesicles (Fig. 29). The diameter of isolated PASM-positive smooth vesicles is 131 ± 32 nm, which compares favorably with the 152 ± 24 nm diameter of PASM-positive cytoplasmic vesicles in intact hyphae (Fig. 21). The characteristic matrix of the cytoplasmic vesicles is not always evident in the isolated membranes, however, and many (perhaps most) of the isolated PASM-positive vesicles must remain unidentified.

The PTA-CrO_3 stain, which has proven to be relatively specific for Achlya plasma membranes in situ (Fig. 19), does not selectively stain any great number of isolated membranes (Fig. 30). This judgement is a relative one, which is based on a comparison between the staining of

Fig. 26. Section of a purified membrane fraction from an A. ambisexualis mycelial homogenate, which contains dictyosome cisternae (D) and unidentified membrane vesicles. x 25,000.

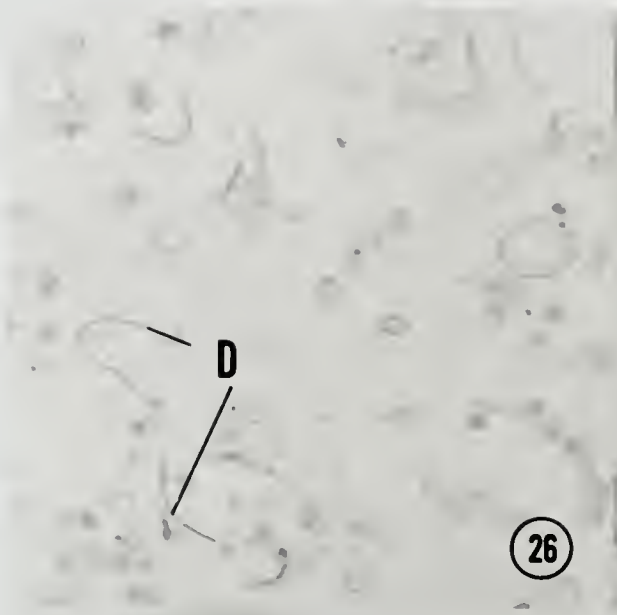
Fig. 27. Section of a purified membrane fraction from an A. ambisexualis mycelial homogenate, which shows a fragmented dictyosome cisterna (D), bearing incipient vesicles. x 77,000.

Fig. 28. Section of a purified membrane fraction from an A. ambisexualis mycelial homogenate, which shows ribosomes (Ri) associated with isolated membranes. x 213,000.

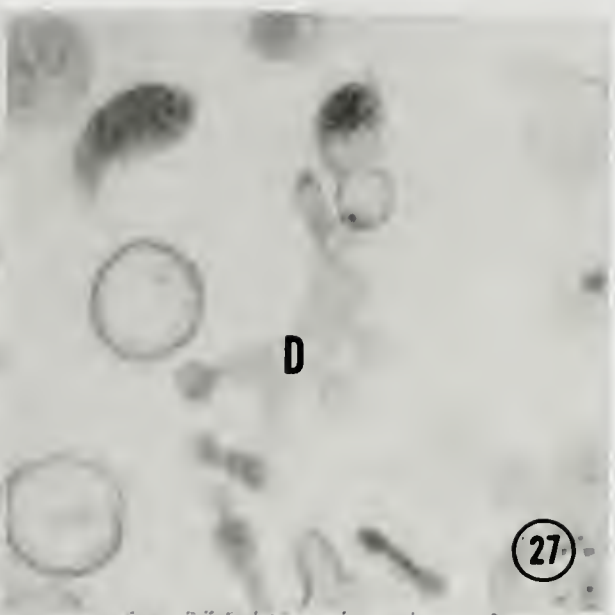
Fig. 29. Section of a purified membrane fraction from an A. ambisexualis mycelial homogenate, which shows the stainability of dictyosome cisternae (D) and some of the unidentified membrane vesicles with the PASM stain for carbohydrate. x 24,000.

Fig. 30. Section of a purified membrane fraction from an A. ambisexualis mycelial homogenate, which shows the lack of stainability of isolated membranes with the PTA-CrO_3 stain. (Compare to Fig. 31.) x 24,000.

Fig. 31. Section of a purified membrane fraction from an A. ambisexualis mycelial homogenate, which has been oxidized with periodic acid, but not stained with PTA-CrO_3. x 24,000.

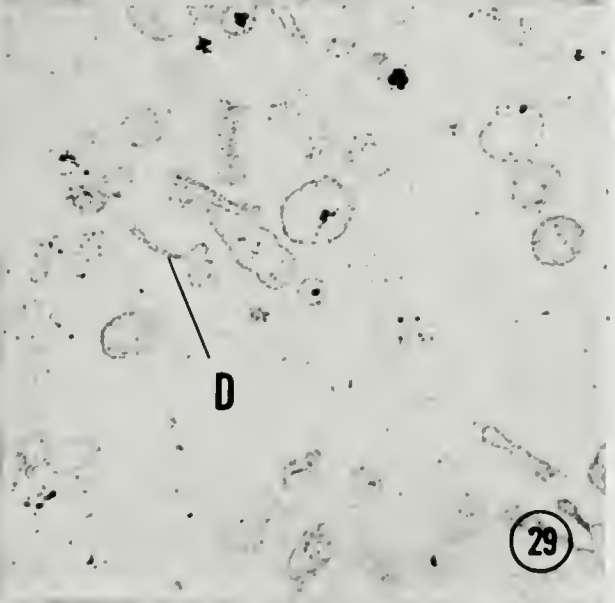


26



27

28



29

30

31

the unidentified smooth vesicles and the staining of dictyosome cisternae, which do not stain in the intact cell. Differences are not seen. Furthermore, the electron-density of PTA-CrO_3-stained isolated membranes is not increased in comparison to control sections (Fig. 31), which were oxidized with periodic acid, but were not stained with PTA-CrO_3. Therefore, no evidence is provided by this test to indicate that any significant proportion of these membranes is composed of plasma membranes or PTA-CrO_3-positive cytoplasmic vesicles.

It is clear, then, that the enrichment of cellulase-rich membranes has not yielded a homogeneous preparation of cellular particles. Indeed, at least three types of particles remain: dictyosome cisternae, PASM-positive smooth vesicles, and PASM-negative smooth vesicles.

Discussion

Most enzyme activities investigated display a distinct maximum coinciding with one of the three peaks of absorbance at 280 nm; however, in each case, some activity is found over a wide range of densities. The materials equilibrating at 1.22 g/cm^3 apparently consist mainly of mitochondria and/or mitochondrial fragments. Of the enzymes studied, only cytochrome oxidase is preferentially located here. In addition, the sedimentability of cytochrome oxidase by low speed centrifugation indicates that the particles in question are relatively large, like mitochondria. This distinctive sedimentation velocity permits mitochondrial contaminants to be eliminated from other membranes of interest.

The isopycnic density of mitochondria in these preparations is unusual, compared to the behavior of mitochondria from other organisms.

For example, mitochondria of higher plants commonly equilibrate at 1.18-1.19 g/cm^3 (e.g., Ray et al., 1969; Moore and Beevers, 1974). In yeast, the value is about 1.12 g/cm^3 (Matile et al., 1971), and in the filamentous fungus Aspergillus tamarii Kita, the value is about 1.19 g/cm^3 (Graves et al., 1976). Thus, Achlya mitochondria equilibrating at 1.22 g/cm^3 do so at a much higher density than do mitochondria from other sources, and it is reasonable to suppose that no greater degree of conformity can be expected of the other organelles. Identification of organelles by their equilibrium densities will, therefore, not be attempted.

Several of the enzyme activities equilibrating at 1.19 g/cm^3 or at 1.15 g/cm^3 show significant activity at both loci. To a greater or lesser degree, the membranes at 1.15 g/cm^3 serve as secondary carriers for those enzymes exhibiting primary activity at 1.19 g/cm^3. The two enzyme activities exhibiting primary activity at 1.15 g/cm^3 (glucose-6-phosphatase and β-glucosidase) likewise show strong secondary activity at 1.19 g/cm^3. Thus, there seems to be a strong degree of relationship between materials at these two loci. Cell compartments exhibiting a gradation of enzyme activities are characteristic of the components of the "endomembrane system" (Morré et al., 1971). This term denotes those cell compartments involved in the transport of materials from their sites of synthesis to their sites of utilization, which may be the exterior of the cell. Depending on the organism, these compartments may include the ER, Golgi apparatus, various vesicles, and the plasma membrane.

Beyond their enzyme activity and isopycnic density, membranes equilibrating at 1.15 g/cm^3 were not further investigated. Glucose-6-phosphatase, which shows maximal activity at this site, is a marker

enzyme for the endoplasmic reticulum in some systems (Fleischer and Kervina, 1974), though this has not been demonstrated in Achlya. Furthermore, the particles in question would seem to be among the lightest in the homogenate, because materials in the two denser peaks can be preferentially sedimented by centrifugation at 25,000 x g x 10 min. These particles may, therefore, be fragments of endoplasmic reticulum.

Cellulase activity, though it is found in virtually every fraction of the gradient, exhibits a clear maximum at 1.19 g/cm^3. A secondary "shoulder" of activity is associated with materials at 1.15 g/cm^3, and this distribution is mimicked by carbohydrate content and by the activities of IDPase, ATPase, and UDPG transferase. The isopycnic density of these membranes can be compared to that of apparently similar membranes from Saprolegnia. UDPG transferase (assayed at pH 5.8) shows primary activity at the 1.13-1.15 g/cm^3 interface of a discontinuous density gradient and secondary activity at the 1.17-1.19 g/cm^3 interface (Fèvre and Dumas, 1977). These two fractions correspond to the 1.15 g/cm^3 and 1.19 g/cm^3 peaks, respectively, for membranes of Achlya in continuous gradients, but the relative intensities of transferase activity in the two peaks is reversed (cf. Fig. 24A). This could be caused by the assay conditions; for example, Van Der Woude et al. (1974) reported that differences in UDPG concentration may be responsible for conflicting reports of the location of transferase activity in different organelles of higher plants.

In Saprolegnia, cellulase and β-1,3-glucanase showed highest activity in a 1.13-1.16 g/cm^3 gradient interface (Fèvre, 1977). This is unlike the behavior of cellulase-rich membranes from Achlya, which

show maximal activity at 1.19 g/cm^3. The reason for the difference is not apparent.

The great variety of enzyme activities localized in the 1.19 g/cm^3 absorbance peak and the fact that UV scans occasionally reveal complexity in this region indicate that more than one type of particle may equilibrate at this density. This and the apparently considerable overlap of contamination from nearby peaks made it imperative that further purification of 1.19 g/cm^3 particles be attempted before speculation could be made concerning the identity (identities) of particles located here. Clearly, contamination of cellulase-rich membranes is reduced by these efforts, as revealed by the steady increase of cellulase specific activity with each step. Carbohydrate content and the specific activities of IDPase, ATPase, and UDPG transferase exhibit similar degrees of enrichment, i.e., about 42 to 56% above the level in the "25K x g" differential fraction (Table 19).

The almost identical coequilibration of membrane-bound carbohydrate and the activities of cellulase, UDPG transferase, ATPase, and IDPase at 1.19 g/cm^3 in density gradients and the steady enrichments of these activities during purification are two lines of evidence indicating that these activities are found primarily in one type of subcellular particle; or, if more than one particle is involved, they are very closely related. There is some precedent in other fungal systems for assuming that the activities in question are found together in the cell. In yeast (Matile et al., 1971; Cortat et al., 1973), transferases, mannan, and glucanases were found together in ER, vesicles, and plasma membranes, while in Saprolegnia (Fèvre, 1977; Fèvre and Dumas, 1977), the major activities of UDPG transferase and glucanase were found

together in fractions rich in Golgi cisternae and what may be Golgi vesicles. Electron microscopically, these fractions appear quite similar to the isolated membrane fraction from Achlya (Fig. 26).

It is not possible to identify the particle(s) bearing these activities by electron microscopy of isolated membranes, because more than one type of particle is present, and most are unidentifiable. However, comparison of the biochemical results with the results of electron microscopy of intact hyphae indicates that 150 nm cytoplasmic vesicles may be the carriers of the carbohydrate and enzyme activities in question. This is based on the cytochemical demonstration that these structures contain both IDPase and carbohydrate (Figs. 14, 15, 21).

The presence of IDPase in these particles deserves some comment. The activity in question does not conform to the cold-latent IDPase activity that has lately served as a biochemical marker for dictyosomes in plants (Ray et al., 1969). In fact, the cytochemical localization of IDPase in Achlya shows no dictyosomal association (Fig. 16). Cold latency and dictyosomal associations are not universal properties of IDPases, however; e.g., Koehler et al. (1976) reported a nonlatent IDPase that they believed to be associated with plasma membranes of kidney bean abscission zones.

Though the role of IDPase is not known, its regular association with polysaccharide synthesis (Dauwalder et al., 1969; Ray et al., 1969) has spurred speculation. For example, the fact that cold-latent IDPase activity increases as UDPG transferase activity in the same particles declines suggested to Ray et al. (1969) that IDPase activity may result from an alteration of UDPG transferase during its

denaturation. This idea is based in part on the probable requirement that UDPG transferases have a recognition site for nucleotide sugars. Ideas that may be more relevant to the noncold-latent IDPase activity of the type found in Achlya are concerned with the ability of IDPase to metabolize nucleoside diphosphate products and inhibitors of polysaccharide synthesis. For instance, UDP is a product and inhibitor of the reaction catalyzed by Coprinus chitin synthetase, and it has been speculated that IDPase might contribute to the regulation of the process by controlling the level of UDP (de Rousset-Hall and Gooday, 1975). Novikoff et al. (1971), in a study of rat ganglia, speculated that hydrolysis of nucleoside diphosphate products of transferases might serve to drive the reaction in the direction of transfer.

Thus, there is a strong precedent for considering that IDPase and UDPG transferase in Achlya are associated together in the same cellular particles, as well as precedent for the association of transferase activity with carbohydrate and glucanase.

GENERAL DISCUSSION

It has been the object of this research to gather data relating the activity of the enzyme cellulase to growth of Achlya hyphae and to determine the subcellular location of this enzyme activity. A measure of success is reflected in the determination that the secretion of cellulase and the level of mycelial cellulase activity are positively correlated with hyphal growth; that membrane-bound cellulase is found in association with carbohydrate and UDPG transferase, which may be involved in wall synthesis; and that cellulase-containing particles are apparently identical to the IDPase-positive apical vesicles observed by electron microscopy.

A simple model for hyphal growth, which incorporates these observations, would be similar to those more general models which have been previously proposed (Park and Robinson, 1966; Grove et al., 1970; Bartnicki-Garcia, 1973). In this model for hyphal growth in Achlya, cellulase and UDPG transferase are synthesized in the ER and are transferred to the dictyosomes. Dictyosomes form at least two kinds of vesicles, which contain cellulase and/or UDPG transferase and possibly other synthetic and lytic enzymes. Polysaccharide synthesis begins in certain of these vesicles while they are still attached to the dictyosomes and apparently continues after detachment. Most likely, this polysaccharide consists of precursors of the wall matrix. After dictyosome vesicles migrate to the hyphal apex, the matrix

materials and the wall enzymes are transferred to the cell surface. Here, enzymes associated with the wall serve to initiate cellulose fibril formation and to integrate the matrix and fibrils to form a functional cell wall.

More general models of hyphal growth have required that both lytic and synthetic processes act simultaneously in the hyphal apex. The association of UDPG transferase, cellulase, and carbohydrate together in cytoplasmic vesicles of Achlya hyphae is consistent with these schemes. Since both the isolated membrane fraction and the normal population of cytoplasmic vesicles are heterogeneous, it cannot be stated that the lytic and synthetic functions reside in the same vesicles. The alternative, wherein each is delivered to the apex in separate vesicles (e.g., Meyer et al., 1976), cannot be ruled out; but if this is so, the two kinds of vesicles are so similar that they are inseparable by the techniques employed.

Vesicular UDPG transferase activity may represent activity in transit to the cell surface or activity required to synthesize the carbohydrate present in the vesicles. The resolution of this question must await the analysis both of the vesicular carbohydrate and of the products of the UDPG transferase reaction. Perhaps then it will be known whether this activity is involved in production of wall fibrils, wall matrix, or both.

Lytic enzymes have traditionally been thought to be involved in the maintenance of plasticity of hyphal apices (Park and Robinson, 1966). This would permit the turgor-driven expansion of what would otherwise be a rigid apical dome. The demonstration that cellulases of higher plants can enhance the rate of wall synthesis (Wong et al.,

1977a) suggests a second possible role for cellulases in apical growth: the endohydrolytic generation of free chain ends which can serve as acceptors in the synthetic reaction. This possibility was anticipated by Bartnicki-Garcia (1973), who proposed that hydrolases may serve both to weaken the wall and to provide ends for synthesis. The apparently intimate association between Achlya transferase and cellulase could reflect either this cooperative action or the more traditionally perceived balance between synthesis and plasticity.

That the balance between wall synthesis and lysis in Achlya may be influential in the regulation of growth is indicated by the 72% increase in mycelial cellulase activity and the high rate of cellulase secretion exhibited by growing hyphae in comparison to nongrowing hyphae. In contrast, UDPG transferase activity varies only slightly between growing and nongrowing hyphae. This is a comparable situation to that observed in the auxin-stimulated pea epicotyl, where cellulase activity is considerably higher in growing regions than in nongrowing regions, but transferase activity is essentially unchanged (Maclachlan, 1976). The explanation in peas, as in Achlya, may be that the lytic effect (whether it be plasticization, primer generation, or both) is a rate limiting step in wall synthesis.

How and where cellulase exerts its effect is not known. Since it is most probable that cellulose is synthesized at the cell surface, it is unlikely that cellulase in cytoplasmic vesicles can be functional in the intact cell. Upon fusion of the vesicle membrane with the plasma membrane, cellulase would be transferred to the cell surface. Though it may be that the enzyme serves its function while still bound to the plasma membrane, it is much more likely that it must be released to act.

This assumption is based both on the observation that maximal cellulase activity is observed after solubilization and upon the requirement that the enzymes have access to existing cellulose chains, which would be at some distance from the plasma membrane.

The solubility of the enzyme would also provide a mechanism for temporal and spatial regulation of the enzyme's influence. In time, cellulase molecules would diffuse into the medium from older areas of the wall and no longer be effective. Only at the tips, where new enzymes are constantly supplied, would the concentration of hydrolytic enzymes be sufficient to enhance the rate of growth. If transferase activity is limited by lytic activity, the loss of cellulase from the wall by diffusion may also provide a mechanism for "turning off" synthesis in older regions of the wall. Other mechanisms may rely on the apparently low stability of the transferase enzyme or on the deprivation of substrate, which may also be carried to the tip by vesicles.

Many of the conclusions that can be drawn from this research are tentative and must await the results of further work before alternative explanations can be excluded. Further work is needed in the localization of enzyme activities by electron microscopy. This is needed because there are no certainly identified marker enzymes for most Achlya organelles. Also, efforts should be made to ensure the success of the cytochemical localization of cellulase activity. The basis for the different enzyme activities of the various apical vesicles should be determined, as should the nature and function of the small coated vesicles and the "submural tubules."

The curious matter of room temperature activation/solubilization deserves further attention. In particular, the behavior of cellulase-rich membranes at 37 C should be investigated to determine whether cellulase is solubilized at this temperature and, if it is, why so little activity is recovered. If evidence implicates the action of a second enzyme in cellulase release, it should be identified. Attempts to enhance solubilization with exogenous enzymes would be helpful in this regard.

The characteristics of a number of Achlya enzymes require definition. In the case of cellulase, more information on the optimal conditions for activity of the secreted and intracellular enzymes may help to determine whether or not these are separate isozymes. UDPG transferase products require characterization before the role of the enzyme in vivo can be more certainly known, and the influence of various assay conditions on the nature of the reaction products should be determined. Finally, the role of Achlya cellulase as a potential wall-modifying enzyme should be confirmed by an experiment to demonstrate the enzyme's ability to enhance the rate of wall synthesis by generating new primer ends.

Other enzymes whose fundamental characteristics could be profitably investigated are IDPase (regarding its substrate specificity) and ATPase (regarding its substrate specificity and possible stimulation by cations).

A problem of critical importance to further investigations of the mechanism of hyphal growth in Achlya lies in the poor resolution of cellular particles on isopycnic gradients. This includes both the closeness of the particle peaks and the heterogeneity of materials in

the 1.19 g/cm^3 peak, in which most cellulase activity is found. It should be determined whether variations in the levels of ions and other ingredients in the homogenizing solution can alter the behavior of some of the particles. In particular, the apparently nonspecific adherence of ribosomes to cytoplasmic membranes should certainly be overcome.

One technique which may help in the resolution of materials from the 1.19 g/cm^3 peak is the use of glutaraldehyde during homogenization to stabilize dictyosomes (e.g., Morré, 1971; Ray et al., 1976). The intact dictyosomes should be heavy enough to be separated by differential centrifugation or by velocity centrifugation, leaving only the lighter nondictyosomal particles to equilibrate at 1.19 g/cm^3.

Concerning the association of mycelial enzymes with hyphal growth, the role of alkaline phosphatase deserves attention. The difference between alkaline phosphatase levels in growing and nongrowing mycelia is even greater than the difference between cellulase levels. Whether this implies some regulatory role and whether the enzyme is truly found in the soluble cytoplasm should be determined.

Finally, the effect of antheridiol-induction upon the activities of enzymes of E87 mycelia should be studied. As just mentioned, changes in the activity of alkaline phosphatase should be noted; and changes in cellulase activity should also be reinvestigated, because previous investigations were concerned with the salt-extractable mycelial cellulases, but not the membrane bound activity. It will be important to compare those enzymatic and cytological changes that accompany the modification of existing walls during antheridial branch initiation with those that have been determined in this research to be associated with vegetative hyphal growth.

REFERENCES

Alexopoulos, C. J. 1962. Introductory mycology. 2nd ed. John Wiley and Sons, Inc., New York.

Armentrout, V. N., G. Hänssler, and D. P. Maxwell. 1976. Acid phosphatase localization in the fungus Whetzelinia sclerotiorum. Arch. Microbiol. 107: 7-14.

Aronson, J. M., B. A. Cooper, and M. S. Fuller. 1967. Glucans of Oomycete cell walls. Science 155: 332-335.

Bal, A. K. 1972. Localization of cellulase in plant cells. In Proc. 4th Int. Cong. of Histochem. and Cytochem. pp. 301-302.

Bal, A. K., D. P. S. Verma, H. Byrne, and G. A. Maclachlan. 1976. Subcellular localization of cellulases in auxin-treated pea. J. Cell Biol. 69: 97-105.

Barksdale, A. W. 1960. Interthallic sexual relations in Achlya, a genus of the aquatic fungi. Amer. J. Bot. 47: 14-23.

Barksdale, A. W. 1962. Effect of nutritional deficiency on growth and sexual reproduction of Achlya ambisexualis. Amer. J. Bot. 49: 633-638.

Barksdale, A. W. 1969. Sexual hormones in Achlya and other fungi. Science 166: 831-837.

Barksdale, A. W. 1970. Nutritional and antheridiol-induced branching in Achlya ambisexualis. Mycologia 62: 411-420.

Barstow, W. E. and J. S. Lovett. 1974. Apical vesicles and microtubules in rhizoids of Blastocladiella emersonii: effects of actinomycin D and cycloheximide on development. Protoplasma 83: 103-117.

Bartnicki-Garcia, S. 1968. Cell wall chemistry, morphogenesis, and taxonomy of fungi. Annu. Rev. Microbiol. 22: 87-108.

Bartnicki-Garcia, S. 1973. Fundamental aspects of hyphal morphogenesis. Symp. Soc. Gen. Microbiol. #23. Microbial Differentiation. pp. 245-267.

Bartnicki-Garcia, S. and E. Lippman. 1972. The bursting tendency of hyphal tips of fungi: presumptive evidence for a delicate balance between wall synthesis and wall lysis in apical growth. J. Gen. Microbiol. 73: 487-500.

Bartnicki-Garcia, S. and W. J. Nickerson. 1962. Isolation, composition, and structure of cell walls of filamentous and yeast-like forms of Mucor rouxii. Biochim. Biophys. Acta 58: 102-119.

Bauer, J. D., P. G. Ackermann, and G. Toro. 1968. Bray's clinical laboratory methods. 7th ed. C. V. Moseby Co. St. Louis.

Bell, T. A., J. L. Etchells, and I. D. Jones. 1955. A method for testing cucumber salt-stock brine for softening activity. USDA, ARS Publ. 72-5.

Bhargava, K. S. 1943. Physiological studies of some members of the family Saprolegniaceae. I. Enzyme action. J. Ind. Bot. Soc. 22: 85-99.

Bio-Rad Technical Bulletin #1051. 1977. Bio-Rad Laboratories. Richmond, California.

Bonnett, H. T. and E. H. Newcomb. 1966. Coated vesicles and other cytoplasmic components of growing root hairs of radish. Protoplasma 62: 59-75.

Bracker, C. E. 1968. Ultrastructure of fungi. Annu. Rev. Phytopathol. 5: 343-374.

Bracker, C. E. 1971. Cytoplasmic vesicles in germinating spores of Gilbertella persicaria. Protoplasma 72: 381-397.

Bracker, C. E., J. Ruiz-Herrera, and S. Bartnicki-Garcia. 1976. Structure and transformation of chitin synthetase particles (chitosomes) during microfibril synthesis in vitro. Proc. Nat. Acad. Sci. USA 73: 4570-4574.

Brown, R. M., Jr., W. W. Franke, H. Kleinig, H. Falk, and P. Sitte. 1970. Scale formation in chrysophycean algae. I. Cellulosic and noncellulosic wall components made by the Golgi apparatus. J. Cell Biol. 45: 247-271.

Byrne, H., N. V. Cristou, D. P. S. Verma, and G. A. Maclachlan. 1975. Purification and characterization of two cellulases from auxin-treated pea epicotyls. J. Biol. Chem. 250: 1012-1018.

Cabib, E. and V. Farkaš. 1971. The control of morphogenesis: an enzymatic mechanism for the initiation of septum formation in yeast. Proc. Nat. Acad. Sci. USA 68: 2052-2056.

Cantino, E. C. 1966. Morphogenesis in aquatic fungi. In The fungi, an advanced treatise. Vol. II. Edited by G. C. Ainsworth and A. S. Sussman. Academic Press, New York. pp. 283-337.

Castle, E. S. 1942. Spiral growth and reversal of spiraling in Phycomyces and their bearing on primary wall structure. Amer. J. Bot. 29: 664-672.

Cleland, R. 1968. Auxin and wall extensibility: reversibility of auxin-induced wall-loosening process. Science 160: 192-194.

Cleland, W. W. 1964. Dithiothreitol, a new protective reagent for SH groups. Biochemistry 3: 480-482.

Coker, W. C. 1923. The Saprolegniaceae, with notes on other water molds. The Univ. of N. Carol. Press, Chapel Hill.

Cortat, M., P. Matile, and F. Kopp. 1973. Intracellular localization of mannan synthetase activity in budding baker's yeast. Biochem. Biophys. Res. Commun. 53: 482-489.

Cortat, M., P. Matile, and A. Wiemken. 1972. Isolation of glucanase-containing vesicles from budding yeast. Arch. Mikrobiol. 83: 189-205.

Dargent, R. 1975. Sur l'ultrastructure des hyphes en croissance de l'Achlya bisexualis Coker. Mise en évidence d'une sécrétion polysaccharidique et d'une activité phosphatasique alcaline dans l'appareil de Golgi et au niveau des vésicules cytoplasmiques apicales. C. R. Acad. Sci. Ser. D 280: 1445-1448.

Dargent, R. and J. Denisse. 1976. Sur l'ultrastructure apicale de l'Achlya bisexualis Coker. Mise en évidence de'une activité phosphatasique acide. C. R. Acad. Sci. Ser. D 282: 1601-1604.

Dauwalder, M., W. G. Whaley, and J. E. Kephart. 1969. Phosphatases and differentiation of the Golgi apparatus. J. Cell Sci. 4: 455-497.

Dauwalder, M., W. G. Whaley, and J. E. Kephart. 1972. Functional aspects of the Golgi apparatus. Sub-cellular Biochemistry 1: 225-275.

Davies, P. J. 1973. Current theories on the mode of action of auxin. Bot. Rev. 39: 139-171.

Dick, M. W. 1973. Saprolegniales. In The fungi, an advanced treatise. Vol. 4B. Edited by G. C. Ainsworth, F. K. Sparrow, and A. S. Sussman. Academic Press, New York. pp. 113-144.

Dietrich, S. M. C. 1973. Carbohydrates from the hyphal walls of some Oomycetes. Biochim. Biophys. Acta 313: 95-98.

Durán, A., B. Bowers, and E. Cabib. 1975. Chitin synthetase zymogen is attached to the yeast plasma membrane. Proc. Nat. Acad. Sci. USA 72: 3952-3955.

Emerson, R. 1941. An experimental study of the life cycles and taxonomy of Allomyces. Lloydia 4: 77-144.

Engels, F. M. 1973. Function of Golgi vesicles in relation to cell wall synthesis. I. Isolation of Golgi vesicles. Acta Bot. Neerl. 22: 6-13.

Engels, F. M. 1974. Function of Golgi vesicles in relation to cell wall synthesis in germinating petunia pollen. IV. Identification of cellulose in pollen tube walls and Golgi vesicles by X-ray diffraction. Acta Bot. Neerl. 23: 209-215.

Fan, D.-F. and G. A. Maclachlan. 1966. Control of cellulase activity by indole acetic acid. Can. J. Bot. 44: 1025-1034.

Fèvre, M. 1972. Contribution to the study of the determination of mycelium branching of Saprolegnia monoica Pringsheim. Z. Pflanzenphysiol. 68: 1-10.

Fèvre, M. 1977. Subcellular localization of glucanase and cellulase in Saprolegnia monoica Pringsheim. J. Gen. Microbiol. 103: 287-295.

Fèvre, M. and C. Dumas. 1977. β-glucan synthetases from Saprolegnia monoica. J. Gen. Microbiol. 103: 297-306.

Fleischer, S. and M. Kervina. 1974. Subcellular fractionation of rat liver. In Methods in enzymology. Vol. 31A. Edited by S. Fleischer and L. Packer. Academic Press, New York. pp. 6-41.

Franke, W. W., W. Herth, W. J. Van Der Woude, and D. J. Morré. 1972. Tubular and filamentous structures in pollen tubes: possible involvement as guide elements in protoplasmic streaming and vectorial migration of secretory vesicles. Planta 105: 317-341.

Frantz, C., J.-C. Roland, F. A. Williamson, and D. J. Morré. 1973. Différenciation in vitro des membranes des dictyosomes. C. R. Acad. Sci. Ser. D 277: 1471-1474.

Fultz, S. A. and A. S. Sussman. 1966. Antigenic differences in the surface of hyphae and rhizoids in Allomyces. Science 152: 785-787.

Galliard, T. 1974. Techniques for overcoming problems of lipolytic enzymes and lipoxygenases in the preparation of plant organelles. In Methods in enzymology. Vol. 31A. Edited by S. Fleischer and L. Packer. Academic Press, New York. pp. 520-528.

Girbardt, M. 1969. Die ultrastruktur der apikalregion von pilzhyphen. Protoplasma 67: 413-441.

Gomori, G. 1952. Microscopic histochemistry: principles and practices. University of Chicago Press, Chicago.

Gooday, G. W. 1971. An autoradiographic study of hyphal growth in some fungi. J. Gen. Microbiol. 67: 125-133.

Graves, L. B., Jr., V. N. Armentrout, and D. P. Maxwell. 1976. Distribution of glyoxylate-cycle enzymes between microbodies and mitochondria in Aspergillus tamarii. Planta 132: 143-148.

Griffin, D. H. and C. Breuker. 1969. Ribonucleic acid synthesis during the differentiation of sporangia in the water mold Achlya. J. Bacteriol. 98: 689-696.

Griffin, D. H., W. E. Timberlake, and J. C. Cheney. 1974. Regulation and macromolecular synthesis, colony development, and specific growth rate of Achlya bisexualis during balanced growth. J. Gen. Microbiol. 80: 381-388.

Grove, S. N. and C. E. Bracker. 1970. Protoplasmic organization of hyphal tips among fungi: vesicles and spitzenkörper. J. Bacteriol. 104: 989-1009.

Grove, S. N., C. E. Bracker, and D. J. Morré. 1970. An ultrastructural basis for hyphal tip growth in Pythium ultimum. Amer. J. Bot. 57: 245-266.

Grove, S. N., J. D. Marlowe, and P. J. Szaniszlo. 1972. Isolation and partial chemical characterization of apical secretory vesicles from growing hyphae of the fungus Gilbertella persicaria. J. Cell Biol. 55: 99a. (abstr.)

Gull, K. and A. P. J. Trinci. 1974. Detection of areas of wall differentiation in fungi using fluorescent staining. Arch. Microbiol. 96: 53-57.

Hall, C. B. 1964. Cellulase activity in tomato fruits according to portion and maturity. Botan. Gaz. 125: 156-157.

Harris, P. J. and D. H. Northcote. 1971. Polysaccharide formation in plant Golgi bodies. Biochim. Biophys. Acta 237: 56-64.

Hayat, M. A. 1970. Principles and techniques of electron microscopy. Biological applications. Vol. 1. Van Nostrand Reinhold Co., New York.

Heath, I. B., J. L. Gay, and A. D. Greenwood. 1971. Cell wall formation in the Saprolegniales: cytoplasmic vesicles underlying developing walls. J. Gen. Microbiol. 65: 225-232.

Heiniger, U. and D. P. Delmer. 1977. UDP-glucose:glucan synthetase in developing cotton fibers. II. Structure of the reaction product. Plant Physiol. 59: 719-723.

Hemmes, D. E. and S. Bartnicki-Garcia. 1975. Electron microscopy of gametangial interaction and oospore development in Phytophthora capsici. Arch. Microbiol. 103: 91-112.

Herbert, D., P. J. Phipps, and R. E. Strange. 1971. Chemical analysis of microbial cells. In Methods in microbiology. Vol. 5B. Edited by J. R. Norris and D. W. Ribbons. Academic Press, New York. pp. 209-244.

Hodges, T. K. and R. T. Leonard. 1974. Purification of a plasma membrane-bound adenosine triphosphatase from plant roots. In Methods in enzymology. Vol. 32. Edited by S. P. Colowick and N. O. Kaplan. Academic Press, New York. pp. 392-406.

Horowitz, D. K. and P. J. Russell. 1974. Hormone-induced differentiation of antheridial branches in Achlya ambisexualis: dependence on ribonucleic acid synthesis. Can. J. Microbiol. 20: 977-980.

Hübscher, G. and G. R. West. 1965. Specific assays of some phosphatases in subcellular fractions of small intestinal mucosa. Nature 205: 799-800.

Hugon, J. and M. Borgers. 1968. Fine structural localization of acid and alkaline phosphatase activities in the absorbing cells of the duodenum of rodents. Histochemie 12: 42-66.

Hunsley, D. 1973. Apical wall structure in hyphae of Phytophthora parasitica. New. Phytol. 72: 985-990.

Hunsley, D. and J. H. Burnett. 1970. The ultrastructural architecture of the walls of some hyphal fungi. J. Gen. Microbiol. 62: 203-218.

Hunsley, D. and D. Kay. 1976. Wall structure of the Neurospora hyphal apex: immunofluorescent localization of wall surface antigens. J. Gen. Microbiol. 95: 233-248.

Iten, W. and P. Matile. 1970. Role of chitinase and other lysosomal enzymes of Coprinus lagopus in the autolysis of fruiting bodies. J. Gen. Microbiol. 61: 301-309.

Jennings, D. H., J. D. Thornton, M. F. J. Galpin, and C. R. Coggins. 1974. Translocation in fungi. In S.E.B. Symposium Vol. 28. Transport at the cellular level. Cambridge: At the University Press.

Johnson, B. F. 1968. Dissolution of yeast glucan induced by 2-deoxyglucose. Exp. Cell Res. 50: 692-694.

Johnson, T. W. 1956. The genus Achlya: morphology and taxonomy. Univ. of Michigan Press, Ann Arbor.

Kane, B. E. 1971. Regulatory mechanisms in cellulase synthesis during hyphal morphogenesis in the water mold Achlya. Ph.D. Thesis, Univ. of Florida.

Kane, B. E., J. B. Reiskind, and J. T. Mullins. 1973. Hormonal control of sexual morphogenesis in Achlya: dependence on protein and ribonucleic acid synthesis. Science 180: 1192-1193.

Keegstra, K., K. W. Talmadge, W. D. Bauer, and P. Albersheim. 1973. The structure of plant cell walls. III. A model of the walls of suspension-cultured sycamore cells based on the interconnections of the macromolecular components. Plant Physiol. 51: 188-196.

Klebs, G. 1899. Zur physiologie der fortpflanzung einiger pilze. II. Saprolegnia mixta de Bary. Jahrb. Wiss. Bot. 33: 519-593.

Koehler, D. E., R. T. Leonard, W. J. Van Der Woude, A. E. Linkins, and L. N. Lewis. 1976. Association of latent cellulase activity with plasma membranes from kidney bean abscission zones. Plant Physiol. 58: 324-330.

Košinová, A., V. Farkaš, S. Machala, and Š. Bauer. 1974. Site of mannan synthesis in yeast. An autoradiographic study. Arch. Microbiol. 99: 255-263.

Lamport, D. T. 1970. Cell wall metabolism. Annu. Rev. Plant Physiol. 21: 235-270.

Lamport, D. T. 1974. The role of hydroxyproline-rich proteins in the extracellular matrix of plants. In Macromolecules regulating growth and development. Edited by E. D. Hay, T. J. King, and J. Papaconstantinou. Academic Press, New York. pp. 113-130.

Larson, D. A. 1965. Fine-structural changes in the cytoplasm of germinating pollen. Amer. J. Bot. 52: 139-154.

Lehle, L., F. Bauer, and W. Tanner. 1977. The formation of glycosidic bonds in yeast glycoproteins. Intracellular localization of the reactions. Arch. Microbiol. 114: 77-81.

Lewis, L. N., F. T. Lew, P. D. Reid, and J. E. Barnes. 1970. Isozymes of cellulase in the abscission zone of Phaseolus vulgaris. In Plant growth substances. Edited by D. J. Carr. Springer-Verlag, Berlin. pp. 234-239.

Lewis, L. N. and J. E. Varner. 1970. Synthesis of cellulase during abscission of Phaseolus vulgaris leaf explants. Plant Physiol. 46: 194-199.

Lips, S. H. 1975. Enzyme content of plant microbodies as affected by experimental procedures. Plant Physiol. 55: 598-601.

Maclachlan, G. A. 1976. A potential role for endo-cellulase in cellulose biosynthesis. Applied Polymer Symposium 28: 645-658.

Mahadevan, P. R. and V. R. Mahadkar. 1970. Role of enzymes in growth and morphology of Neurospora crassa: cell-wall-bound enzymes and their possible role in branching. J. Bacteriol. 101: 941-947.

Marriott, M. S. 1975. Enzymic activity of purified plasma membranes from the yeast and mycelial forms of Candida albicans. J. Gen. Microbiol. 89: 345-352.

Martino, C. de and L. Zamboni. 1967. Silver methenamine stain for electron microscopy. J. Ultrastruct. Res. 19: 273-282.

Mascarenhas, J. P. and J. Lafountain. 1972. Protoplasmic streaming, cytochalasin B, and growth of the pollen tube. Tissue and Cell 4: 11-14.

Matile, P. 1971. Vacuoles, lysosomes of Neurospora. Cytobiologie 3: 324-330.

Matile, P., M. Cortat, A. Wiemken, and A. Frey-Wyssling. 1971. Isolation of glucanase-containing particles from budding Saccharomyces cerevisiae. Proc. Nat. Acad. Sci. USA 68: 636-640.

Maxwell, D. P., G. Hänssler, and M. D. Maxwell. 1978. Ultrastructural localization of acid phosphatase in Pythium parvecandrum. Protoplasma 94: 73-82.

McClure, W. K., D. Park, and P. M. Robinson. 1968. Apical organization in the somatic hyphae of fungi. J. Gen. Microbiol. 50: 177-182.

McLaughlin, D. J. 1973. Ultrastructure of sterigma growth and basidiospore formation in Coprinus and Boletus. Can. J. Bot. 51: 145-150.

McMurrough, I., A. Flores-Carreon, and S. Bartnicki-Garcia. 1971. Pathway of chitin synthesis and cellular localization of chitin synthetase in Mucor rouxii. J. Biol. Chem. 246: 3999-4007.

Meyer, D. H. 1976. Secretion of β-glucosidase by Ochromonas danica. Arch. Microbiol. 109: 263-270.

Meyer, R., R. W. Parish, and H. R. Hohl. 1976. Hyphal tip growth in Phytophthora. Gradient distribution and ultrahistochemistry of enzymes. Arch Microbiol. 110: 215-224.

Mills, G. L. and E. C. Cantino. 1978. The lipid composition of the Blastocladiella emersonii γ particle and the function of the γ particle lipid in chitin formation. Exp. Mycol. 2: 99-109.

Mollenhauer, H. H. and D. J. Morré. 1976. Cytochalasin B, but not colchicine, inhibits migration of secretory vesicles in root tips of maize. Protoplasma 87: 39-48.

Moor, H. 1967. Endoplasmic reticulum as the initiator of bud formation in yeast (S. cerevisiae). Arch. Mikrobiol. 57: 135-146.

Moore, T. S. and H. Beevers. 1974. Isolation and characterization of organelles from soybean suspension cultures. Plant Physiol. 53: 261-265.

Morré, D. J. 1971. Isolation of Golgi apparatus. In Methods in enzymology. Vol. 22. Edited by W. B. Jakoby. Academic Press, New York. pp. 130-149.

Morré, D. J., H. H. Mollenhauer, and C. E. Bracker. 1971. Origin and continuity of Golgi apparatus. In Origin and continuity of cell organelles. Edited by J. Reinert and H. Ursprung. Springer-Verlag, New York. pp. 82-126.

Morré, D. J. and W. J. Van Der Woude. 1974. Origin and growth of cell surface components. In Macromolecules regulating growth and development. Edited by E. D. Hay, T. J. King, and J. Papaconstantinou. Academic Press, New York. pp. 81-111.

Mueller, S. C., R. M. Brown, Jr., and T. K. Scott. 1976. Cellulosic microfibrils: nascent stages of synthesis in a higher plant cell. Science 194: 949-951.

Mullins, J. T. 1973. Lateral branch formation and cellulase production in the water molds. Mycologia 65: 1007-1014.

Mullins, J. T. and A. W. Barksdale. 1965. Parasitism of the chytrid Dictyomorpha dioica. Mycologia 57: 352-359.

Mullins, J. T. and E. A. Ellis. 1974. Sexual morphogenesis in Achlya: ultrastructural basis for the hormonal induction of antheridial hyphae. Proc. Nat. Acad. Sci. USA 71: 1347-1350.

Nečas, O. 1971. Cell wall synthesis in yeast protoplasts. Bacteriol. Rev. 35: 146-170.

Nickerson, W. J. and G. Falcone. 1959. Function of protein disulfide reductase in cellular division of yeasts. In Sulfur in proteins. Edited by R. Benesch et al. Academic Press, New York. pp. 409-424.

Nolan, R. A. and A. K. Bal. 1974. Cellulase localization in hyphae of Achlya ambisexualis. J. Bacteriol. 117: 840-843.

Novikoff, A. B. and S. Goldfischer. 1961. Nucleoside-phosphatase activity in the Golgi apparatus and its usefulness for cytological studies. Proc. Nat. Acad. Sci. USA 47: 802-810.

Novikoff, P. M., A. B. Novikoff, N. Quintana, and J.-J. Hauw. 1971. Golgi apparatus, Gerl, and lysosomes of neurons in rat dorsal root ganglia, studied by thick section and thin section cytochemistry. J. Cell Biol. 50: 859-886.

O'Day, D. H. and P. A. Horgen. 1974. The developmental patterns of lysosomal enzyme activities during Ca^{++}-induced sporangium formation in Achlya bisexualis. I. Acid phosphatase. Devel. Biol. 39: 116-124.

Olson, A. C., J. Bonner, and D. J. Morré. 1965. Force extension analysis of Avena coleoptile cell walls. Planta 66: 126-134.

Ordin, L. and M. A. Hall. 1968. Cellulose synthesis in higher plants from UDP glucose. Plant Physiol. 43: 473-476.

Ott, D. W. and R. M. Brown, Jr. 1974. Developmental cytology of the genus Vaucheria. I. Organization of the vegetative filament. Br. Phycol. J. 9:111-126.

Palade, G. 1975. Intracellular aspects of the process of protein synthesis. Science 189: 347-358.

Parish, R. W. 1975. The lysosome-concept in plants. II. Location of acid hydrolases in maize root tips. Planta 123: 15-31.

Park, D. and P. M. Robinson. 1966. Aspects of hyphal morphogenesis in fungi. In Trends in plant morphogenesis. Edited by E. G. Cutter. John Wiley and Sons, Inc., London. pp. 27-44.

Parker, B. C., R. D. Preston, and G. E. Fogg. 1963. Studies of the structure and chemical composition of the cell walls of Vaucheriaceae and Saprolegniaceae. Proc. Roy. Soc. London Ser. B 158: 435-445.

Pettersson, G. 1963. Separation of cellulases on sephadex G-100. Biochim. Biophys. Acta 77: 665-667.

Pickett-Heaps, J. D. 1968. Further ultrastructural observations on polysaccharide localization in plant cells. J. Cell Sci. 3: 55-64.

Preston, R. D. 1974. The physical biology of plant cell walls. Chapman and Hall, London.

Raper, J. R. 1951. Sexual hormones in Achlya. Amer. Scientist 39: 110-120[130].

Ray, P. M. 1973. Regulation of β-glucan synthetase activity by auxin in pea stem tissue. I. Kinetic aspects. Plant Physiol. 51: 601-608.

Ray, P. M., W. R. Eisinger, and D. G. Robinson. 1976. Organelles involved in cell wall polysaccharide formation and transport in pea cells. Ber. Deutsch. Bot. Ges. 89: 121-146.

Ray, P. M., T. L. Shininger, and M. M. Ray. 1969. Isolation of β-glucan synthetase particles from plant cells and identification with Golgi membranes. Proc. Nat. Acad. Sci. USA 64: 605-612.

Reese, E. T. 1959. Cellulose decomposition: fungi. In Marine boring and fouling organisms. Edited by D. L. Ray. Univ. of Washington Press, Seattle. pp. 265-300.

Reid, E. and R. Williamson. 1974. Centrifugation. In Methods in enzymology. Vol. 31A. Edited by S. Fleischer and L. Packer. Academic Press, New York. pp. 713-733.

Reid, P. D., H. G. Strong, F. Lew, and L. N. Lewis. 1974. Cellulase and abscission in the red kidney bean (Phaseolus vulgaris). Plant Physiol. 53: 732-737.

Reynolds, E. S. 1963. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell Biol. 17: 208-212.

Robertson, N. F. 1965. The mechanism of cellular extension and branching. In The fungi, an advanced treatise. Vol. I. Edited by G. C. Ainsworth and A. S. Sussman. Academic Press, New York. pp. 613-623.

Robinson, D. G. and W. R. Cummins. 1976. Golgi apparatus secretion in plasmolyzed Pisum sativum L. Protoplasma 90: 369-379.

Robinson, D. G., W. R. Eisinger, and P. M. Ray. 1976. Dynamics of the Golgi system in wall matrix polysaccharide synthesis and secretion by pea cells. Ber. Deutsch. Bot. Ges. 89: 147-161.

Roggen, H. P. J. R. and R. G. Stanley. 1969. Cell-wall-hydrolyzing enzymes in wall formation as measured by pollen tube extension. Planta 84: 295-303.

Roland, J.-C., C. A. Lembi, and D. J. Morré. 1972. Phosphotungstic acid-chromic acid as a selective electron-dense stain for plasma membranes of plant cells. Stain Techn. 47: 195-200.

Rosen, W. G., S. R. Gawlik, W. V. Dashek, and K. A. Siegesmund. 1964. Fine structure and cytochemistry of Lilium pollen tubes. Amer. J. Bot. 51: 61-71.

Rousset-Hall, A. de and G. W. Gooday. 1975. A kinetic study of a solubilized chitin synthetase preparation from Coprinus cinereus. J. Gen. Microbiol. 89: 146-154.

Ruesink, A. W. 1969. Polysaccharidases and the control of cell wall elongation. Planta 89: 95-107.

Ruiz-Herrera, J. and S. Bartnicki-Garcia. 1974. Synthesis of cell wall microfibrils in vitro by a "soluble" chitin synthetase from Mucor rouxii. Science 186: 357-359.

Ruiz-Herrera, J. and S. Bartnicki-Garcia. 1976. Proteolytic activation and inactivation of chitin synthetase from Mucor rouxii. J. Gen. Microbiol. 97: 241-249.

Ruiz-Herrera, J., V. O. Sing, W. J. Van Der Woude, and S. Bartnicki-Garcia. 1975. Microfibril assembly by granules of chitin synthetase. Proc. Nat. Acad. Sci. USA 72: 2706-2710.

Runyon, R. P. and A. Haber. 1971. Fundamentals of behavioral statistics. 2nd ed. Addison-Wesley Publishing Co., Reading, Mass.

Satir, B., C. Schooley, and P. Satir. 1973. Membrane fusion in a model system. Mucocyst secretion in Tetrahymena. J. Cell Biol. 56: 153-176.

Sentandreu, R. and D. H. Northcote. 1969. The formation of buds in yeast. J. Gen. Microbiol. 55: 393-398.

Shore, G. and G. A. Maclachlan. 1973. Indoleacetic acid stimulates cellulose deposition and selectively enhances certain β-glucan synthetase activities. Biochim. Biophys. Acta 329: 271-282.

Shore, G. and G. A. Maclachlan. 1975. The site of cellulose synthesis. Hormone treatment alters the intracellular location of alkali-insoluble β-1,4-glucan (cellulose) synthetase activities. J. Cell Biol. 64: 557-571.

Shore, G., Y. Raymond, and G. A. Maclachlan. 1975. The site of cellulose synthesis. Cell surface and intracellular β-1,4-glucan (cellulose) synthetase activities in relation to the stage and direction of cell growth. Plant Physiol. 56: 34-38.

Siegel, S. M. 1968. Biochemistry of the plant cell wall. In Comprehensive biochemistry. Vol. 26A. Edited by M. Florkin and E. H. Stotz. Academic Press, New York. pp. 1-51.

Sietsma, J. H., J. J. Child, L. R. Nesbitt, and R. H. Haskins. 1975. Chemistry and ultrastructure of the hyphal walls of Pythium acanthicum. J. Gen. Microbiol. 86: 29-38.

Sietsma, J. H. and R. H. Haskins. 1968. The incorporation of cholesterol by Pythium sp PRL 2142, and some of its effects on cell metabolism. Can. J. Biochem. 46: 813-818.

Sievers, A. 1967. Elektronmikroskopische untersuchungen zur geotropische reaktion. II. Die polare organisation des normal wachsenden rhizoids von Chara foetida. Protoplasma 64: 225-253.

Singer, S. J. 1974. The molecular organization of membranes. Annu. Rev. Biochem. 43: 805-833.

Smith, J. H. 1923. On the apical growth of fungal hyphae. Ann. Bot. 37: 341-343.

Southworth, D. and D. B. Dickinson. 1975. β-1,3-glucan synthase from Lilium longiflorum pollen. Plant Physiol. 56: 83-87.

Sparrow, F. K., Jr. 1960. Aquatic phycomycetes. 2nd ed. The Univ. of Michigan Press, Ann Arbor.

Spatz, L. and P. Strittmatter. 1973. A form of reduced nicotinamide adenine dinucleotide-cytochrome b_5 reductase containing both the catalytic site and an additional hydrophobic membrane-binding segment. J. Biol. Chem. 248: 793-799.

Spencer, F. S., B. Ziola, and G. A. Maclachlan. 1971. Particulate glucan synthetase activity: dependence on acceptor, activator, and plant growth hormone. Can. J. Biochem. 49: 1326-1332.

Taussky, H. H. and E. Shorr. 1953. A microcolorimetric method for the determination of inorganic phosphorus. J. Biol. Chem. 202: 675-685.

Terra, N. de and E. L. Tatum. 1961. Colonial growth in Neurospora. Science 134: 1066-1068.

Thomas, D. des S. 1966. Hormone-enzyme regulation of morphogenesis in the water mold Achlya ambisexualis Raper. Ph.D. Thesis, Univ. of Florida.

Thomas, D. des S. 1970. Effects of water stress on induction and secretion of cellulase and on branching in the water mold Achlya ambisexualis. Can. J. Bot. 48: 977-979.

Thomas, D. des S. and J. T. Mullins. 1967. Role of enzymatic wall softening in plant morphogenesis: hormonal induction in Achlya. Science 156: 84-85.

Thomas, D. des S. and J. T. Mullins. 1969. Cellulase induction and wall extension in the water mold Achlya ambisexualis. Physiol. Plant. 22: 347-353.

Thomas, D. des S., J. E. Smith, and R. G. Stanley. 1969. Stereo-specific regulation of plant glucan synthetase in vitro. Can. J. Bot. 47: 489-496.

Timberlake, W. E. 1976. Alterations in RNA and protein synthesis associated with steroid hormone-induced sexual morphogenesis in the water mold Achlya. Devel. Biol. 51: 202-214.

Timberlake, W. E., L. McDowell, J. Cheney, and D. H. Griffin. 1973. Protein synthesis during differentiation of the sporangia in the water mold Achlya. J. Bacteriol. 116: 67-73.

Tolbert, N. E. 1974. Isolation of subcellular organelles of metabolism on isopycnic sucrose gradients. In Methods in enzymology. Vol. 31A. Edited by S. Fleischer and L. Packer. Academic Press, New York. pp. 734-746.

Tsai, C. M. and W. Z. Hassid. 1971. Solubilization and separation of uridine diphospho-D-glucose:β-(1→4) glucan and uridine diphospho-D-glucose:β-(1→3) glucan glucosyltransferases from coleoptiles of Avena sativa. Plant Physiol. 47: 740-744.

Tsai, C. M. and W. Z. Hassid. 1973. Substrate activation of β-(1→3) glucan synthetase and its effect on the structure of β-glucan obtained from UDP-D-glucose and particulate enzyme of oat coleoptiles. Plant Physiol. 51: 998-1001.

Turian, G. 1976. Reducing power of hyphal tips and vegetative apical dominance in fungi. Experientia 32: 989-991.

Van Der Woude, W. J., C. A. Lembi, and D. J. Morré. 1972. Auxin (2,4-D) stimulation (in vivo and in vitro) of polysaccharide synthesis in plasma membrane fragments isolated from onion stems. Biochem. Biophys. Res. Comm. 46: 245-253.

Van Der Woude, W. J., C. A. Lembi, D. J. Morré, J. I. Kindinger, and L. Ordin. 1974. β-glucan synthetases of plasma membrane and Golgi apparatus from onion stem. Plant Physiol. 54: 333-340.

Van Der Woude, W. J. and D. J. Morré. 1968. Endoplasmic reticulum-dictyosome-secretory vesicle associations in pollen tubes of Lilium longiflorum Thunb. Proc. Indiana Acad. Sci. 77: 164-170.

Van Der Woude, W. J., D. J. Morré, and C. E. Bracker. 1971. Isolation and characterization of secretory vesicles in germinated pollen of Lilium longiflorum. J. Cell Sci. 8: 331-351.

Vian, B. and J.-C. Roland. 1972. Différenciation des cytomembranes et renouvellement du plasmalemme dans les phènoménes de sécrétions végétales. J. Microscopie 13: 119-136.

Wang, M. C. and S. Bartnicki-Garcia. 1966. Biosynthesis of β-1,3- and β-1,6-linked glucan by Phytophthora cinnamomi hyphal walls. Biochem. Biophys. Res. Comm. 24: 832-837.

Wattiaux, R. 1969. Biochemistry and function of lysosomes. In Handbook of molecular cytology. Edited by A. Lima-de-Faria. John Wiley and Sons, Inc., New York. pp. 1159-1178.

Wessels. J. G. H. 1966. Control of cell-wall glucan degradation during development in Schizophyllum commune. Anton. Leeuwenhoek J. Microbiol. 32: 341-355.

Whiffen, A. J. 1945. Nutritional studies of representatives of five genera in the Saprolegniaceae. J. Elisha Mitchell Scient. Soc. 61: 114-123.

Whitaker, D. R. 1963. Criteria for characterizing cellulases. In Advances in enzymic hydrolysis of cellulose and related materials. Edited by E. T. Reese. The Macmillan Co., New York.

Whitaker, D. R. 1971. Cellulases. In The enzymes. 3rd ed. Vol. 5. Edited by P. D. Boyer. Academic Press, New York. pp. 273-290.

White, A., P. Handler, E. L. Smith, and DeW. Stetten, Jr. 1959. Principles of biochemistry. 2nd ed. McGraw-Hill Book Co., Inc., New York.

Wong, Y.-S., G. B. Fincher, and G. A. Maclachlan. 1977a. Cellulases can enhance β-glucan synthesis. Science 195: 679-681.

Wong, Y.-S., G. B. Fincher, and G. A. Maclachlan. 1977b. Kinetic properties and substrate specificities of two cellulases from auxin-treated pea epicotyls. J. Biol. Chem. 252: 1402-1407.

Wood, T. M. 1968. Cellulolytic enzyme system of Trichoderma koningii. Separation of components attacking native cotton. Biochem. J. 109: 217-227.

Wooding, F. B. P. 1968. Radioautographic and chemical studies of incorporation into sycamore vascular tissue walls. J. Cell Sci. 3: 71-80.

Zalokar, M. 1965. Integration of cellular metabolism. In The fungi, an advanced treatise. Vol. I. Edited by G. C. Ainsworth and A. S. Sussman. Academic Press, New York. pp. 377-426.

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Mr. Hill is married to the former Evalie Dian Gallup.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

J. Thomas Mullins, Chairman
Professor of Botany

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Henry C. Aldrich
Professor of Microbiology and Cell Science

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This dissertation was submitted to the Graduate Faculty of the Department of Botany in the College of Liberal Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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